THE PINEAL GLAND AND REPRODUCTION IN THE TAMMAR WALLABY, *MACROPUS EUGENII*.

by

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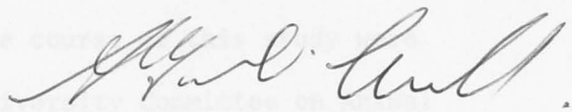
This thesis is my own work except where acknowledged otherwise.



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CONTENTS

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ABSTRACT

The female tammar, *Macropus eugenii*, is a strict seasonal breeding marsupial, and photoperiod is the proximal environmental factor regulating its seasonality. As the pineal gland has been shown to mediate the effects of photoperiod on reproduction in eutherian mammals, this study examined whether a similar role could be assigned to the tammar pineal.

Techniques for pinealectomy and superior cervical ganglionectomy were developed, validated, and applied to determine the role of the pineal in seasonal reproduction. A radioimmunoassay that measured plasma concentrations of melatonin, a pineal hormone, was also validated for use in the tammar.

Plasma levels of melatonin were highest during the dark phase of the daily light/dark cycle. Pinealectomy or ganglionectomy abolished this nocturnal rise, indicating that the pineal is the major source of melatonin in this species.

The duration of the nocturnal melatonin rise was dependent on the duration of the dark phase, and so changed with changes in the photoperiod. A decrease in daylength increased the duration of the nocturnal rise. This initiates reactivation of the quiescent corpus luteum and blastocyst, as melatonin injections that mimic the increase in duration also induce reactivation. This suggests that seasonal changes in photoperiod regulate the annual reproductive cycle of the

tammar via the pineal, and this is effected by seasonal changes in the duration of the nocturnal melatonin rise.

However, the pineal is not totipotent in its regulation of the annual reproductive cycle. After pinealectomy or ganglionectomy, seasonality was abolished in approximately half of the treated animals, but retained in the remainder. Although the influence of other geophysical cues could not be dismissed, and there is some evidence that pinealectomized tammars retained photosensitivity, it is proposed that the tammar possesses an endogenous circannual rhythm of reproduction.

This study has shown that both pineal mediated photoperiod information, and an endogenous circannual rhythm, control seasonal reproduction in the tammar.

LIST OF TABLES AND FIGURES

Chapter 4

Chapter 5

ABBREVIATIONS.

CL	corpus luteum
E.S.T.	Eastern Standard Time
FSH	follicle-stimulating hormone
LH	luteinizing hormone
LH-RH	luteinizing hormone-releasing hormone
ml	millilitre
ng	nanogram
pg	picogram
PINX	pinealectomy
PY	pouch young
RIA	radioimmunoassay
RPY	remove pouch young
SCGX/ganglionectomy	bilateral superior cervical ganglionectomy
s.d.	standard deviation
s.e.m.	standard error of the mean
Sham PINX	sham pinealectomy
Sham SCGX	sham bilateral superior cervical ganglionectomy
15L:9D	15 hours of light and 9 hours of dark per 24 hour day.

CHAPTER ONE

THE PINEAL GLAND

1.1 An historical perspective

1.2 Anatomy and physiology of the mammalian pineal gland

1.3 Effects of pineal mediated photoperiod on mammalian reproduction

1.4 Mode of pineal influence on the reproductive axis

- (i) Melatonin - the putative pineal hormone
- (ii) Target tissues of melatonin
- (iii) Mode of influence of melatonin
- (iv) Models of reproductive seasonality

1.5 The Marsupials

- (i) Marsupial pineal anatomy
- (ii) Marsupial pineal physiology
- (iii) Photoperiod effects on reproduction

1.6 The reproductive biology of the tammar, *Macropus eugenii*

- (i) Introduction
- (ii) The pregnant and oestrous cycles
- (iii) The annual reproductive cycle
- (iv) Evidence for the involvement of photoperiod and the pineal

1.7 The scope and aims of this thesis.

THE PINEAL GLAND

1.1 An historical perspective

Until recently the function of the pineal gland has been unclear. Historically, it has been variously described as a valve controlling memory flow (Herophilus and Erasistradus, c.300 B.C.), the seat of the soul (Descartes, 1640) or if 'diseased', a cause of insanity (Morgagni, 1761) (c.f. Altschule, 1975). The pineal is now known to be intimately related to environmental photoperiod, and this relationship shows phylogenetic change. The pineal (*epiphysis cerebri*) of the Agnatha and Reptilia has photoreceptor qualities (Dodt, 1973; Hamasaki and Eder, 1977), whilst in the Aves it has been regarded as a photoendocrine transducer (Gaston and Menaker, 1968; Hisano *et al.*, 1972). In the Mammalia the term 'neuroendocrine transducer' has been proposed (Wurtman and Axelrod, 1965). However, whilst these classical descriptions provide an indication of differences between the vertebrate classes, they are now regarded as too restrictive by some workers (Kappers, 1981).

As many aspects of an animal's physiology and behaviour are dictated by environmental lighting, it is not surprising that the pineal has now been found to be involved in these processes. However, as the response to photoperiod varies among species, and can change with age, early studies of pineal function were confounded. The classical approach to endocrinology of removing the supposed gland often had no effect or, at best, the results were ambiguous. This

led many to suspect that in mammals the pineal was a vestigial structure with little physiological importance. However from the few studies that yielded unambiguous results, Kitay and Altschule (1954b) concluded the pineal was involved, in some way, in gonadal function and some other physiological processes.

The recent rediscovery of the pineal gland has resulted in voluminous reports on the control of pineal function, and its role in many aspects of vertebrate physiology and behaviour. Inclusion in this chapter of an appraisal of the effects of the pineal in all of these aspects, however brief, may distract the reader from the essence of the subject of this thesis. For this reason I have restricted this review primarily to the control of pineal function, and its role in mammalian reproduction, concentrating mainly on those works I feel have contributed most, or given new direction, to these areas of pinealogy.

The review will consider the following:

- mammalian pineal anatomy and physiology,
- evidence that the effects of photoperiod on mammalian reproduction are mediated by the pineal,
- how the pineal mediates these effects, and
- evidence for photoperiod/pineal effects on reproduction in marsupials.

1.2 Anatomy and physiology of the mammalian pineal gland

Embryologically the pineal develops as an outgrowth of the posterior dorsal wall of the diencephalon anterior to the posterior commissure (Quay, 1974). In most adult mammals studied, the pineal

is a solid or saccular structure connected to the habenular commissure at its anterior base and the posterior commissure at the posterior base. It usually lies close to the point of its embryonic origin. In rodents, however, variable amounts of pineal tissue are found from the site of origin extending to beneath the top of the skull, being connected by a stalk when in the more superficial aspects (Quay, 1965, 1974).

Two types of pineal cells are now recognised, and the presence of a third type has not been ruled out. The first type of cell, characterized by the presence of granular vesicles (GV), is the pinealocyte. It is derived phylogenetically from the neurosensory photoreceptor cells of submammalian vertebrates. The GV probably represent discrete accumulations of secretory products (Pévet, 1981). Sub-populations of pinealocytes appear to exist (Pévet, 1981) and cytological responses to photoperiod are reported to differ between basal and more superficial pinealocytes in the White-footed mouse (Quay, 1956). The second pineal cell type lacks GV and is regarded as an interstitial or glial cell. Pigment-containing cells in some species have been described in the presence of glial cells. These may represent a third pineal cell type (Pévet, 1981).

A relationship between environmental light and the pineal was demonstrated by Quay (1956) and Fiske, Bryant and Putnam (1960). They showed that histological and morphological changes in the pineals of rodents were associated with changes in photoperiod. Circadian biochemical changes in the pineal were shown subsequently in the rat (Quay, 1963), as pineal serotonin levels were high during the light phase compared with the dark phase. The activity of the enzymes

serotonin N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT), responsible for melatonin synthesis, was shown to be increased during dark and suppressed by light (Wurtman, Axelrod and Phillips, 1963; Klein and Weller, 1970), thereby accounting for diurnal variations in pineal melatonin content.

Although a relationship between photoperiod and pineal function was demonstrated, the retinal cone-like photoreceptor cells of the pineals of lower vertebrates, failed to be identified in the adult mammalian pineal. In the search for an anatomical pathway mediating the demonstrated effects of photoperiod on pineal function, Quay (1961) showed the involvement of the eyes. Orbital enucleation of rats prevented the decrease in pineal weight observed under constant illumination. Blinding also abolished the light-dependent fluctuations in pineal HIOMT (Snyder, Zweig, Axelrod and Fischer, 1965). As post-retinal neural information appeared to be involved, studies of the innervation of the pineal were undertaken.

Kappers (1960, 1965) showed the rat pineal received sympathetic fibres from the superior cervical ganglia (SCG) but parasympathetic fibres have also been described in other species (Romijn, 1975). A functional relationship between the superior cervical ganglia (SCG) and pineal was demonstrated by the abolition of the response of HIOMT activity to photoperiod (Wurtman, Axelrod and Fischer, 1964), and the circadian pineal serotonin rhythm (Fiske, 1964), following ganglionectomy (SCGX).

The proximal sympathetic innervation of the mammalian pineal was shown to be by either 1) a diffuse system where small fibres accompany blood vessels entering the organ, or 2) a more consolidated system where these fibres form the *nervi conarii*. The latter systemwas described in rodents and primates (Quay, 1974). No direct connection between the brain and pineal were originally thought to exist. Nerve fibres in the pineal originating from the habenular and posterior commissures were considered aberrant fibre loops lacking synaptic connections with the pineal (Kappers, 1965). However, direct anatomical connections between the habenular and pineal were subsequently reported to occur in ferrets, cats, and monkeys (David and Herbert, 1973; Nielsen and Møller, 1975). Electrophysiological evidence also indicates habeno-pineal neural connections in rodents (Rønnekleiv, Kelly and Wuttke, 1980). Recently, direct neural connections between the rat pineal and habenular, colliculi, amygdala, paraventricular and suprachiasmatic nuclei, preoptic area and olfactory centers have been demonstrated by retrograde transport of horseradish peroxidase (Guérillot, Pfister, Müller and Da Lage, 1982).

Although detection of photoperiod by the retinae was shown to influence pineal function, the known post-retinal visual projections were not involved. The enzyme NAT was found to exhibit circadian rhythmicity in rat pineals, i.e. in the absence of a daily light-dark cycle the rhythm of NAT became "free-running". This was entrained by a light-dark cycle (Klein and Weller, 1970). After transection of either or both of the primary or accessory optic tracts, however, the pineal NAT rhythm could still be entrained by photoperiod. This suggested that a separate projection to the known visual projections from the retina was controlling pineal function (Moore and Klein, 1974).

A retinohypothalamic projection had been described in mammals (Moore, 1973; Moore and Lenn, 1972) which branched directly off the optic chiasm and terminated bilaterally in the suprachiasmatic nuclei (SCN). Transection of the retinohypothalamic tract (RHT), rostral to the SCN, had no effect on the NAT rhythm. However, bilateral lesions of the SCN, transection of the medial hypothalamus caudal to the SCN, or the medial forebrain bundle abolished the NAT rhythm (Moore and Klein, 1974). From these results a possible central pathway involved in the regulation of the pineal NAT rhythm was proposed: (i) photic stimuli from the retinae reach the SCN via the RHT, (ii) information is relayed through the lateral hypothalamus and brain stem to the intermediolateral cell column of the spinal cord, whose fibres give rise to the preganglionic fibres of the SCG, (iii) postganglionic fibres innervate the pineal. Photic stimulation of the retinae inhibits SCN stimulation of NAT activity and so may entrain the pineal NAT rhythm, generated by the SCN, to the daily photoperiod. In the absence of retinal stimulation, the SCN continue to generate rhythmic NAT activity. The SCN were therefore considered to be the "central rhythm generator" of pineal function (Moore and Klein, 1974).

1.3 Effects of pineal mediated photoperiod on mammalian reproduction.

Although a relationship between environmental light and reproduction was well recognized by the 1940s, how this was mediated was not understood.

In the rat, constant light advanced sexual maturation, reflected in enlargement of the gonads and changes in pituitary gonadotrophin content (Browman, 1937; Fiske, 1941). Bissonnette (1932) showed that

ferrets would become reproductively active during the normal anoestrous period in winter if they were exposed to extra hours of light per day. This response was impaired after division of the optic nerves (Bissonnette, 1935a, b), so in an attempt to localize the postretinal visual centres mediating this reproductive response to extra light, Clark, McKeown and Zuckerman, (1939),ablated the lateral geniculate body, superior colliculi and visual cortex of ferrets. However,the response persisted in the absence of any one of these structures.

Early studies on the pineal gland had shown that it had 'anti-gonadal' properties. Pinealectomy of young rats caused ovarian hypertrophy and this could be prevented by the simultaneous administration of pineal extracts (Kitay and Altschule, 1954; Wurtman, Altschule and Holmgren, 1959). These findings, together with the demonstrated relationship between environmental light and the pineal (Quay, 1956; Fiske *et al.*, 1960), suggested that photoperiod influences on reproduction may be mediated by the pineal. Hoffman and Reiter (1965a, b) showed this to be true in the hamster, as pinealectomy prevented testicular atrophy in animals exposed to short photoperiods.

The pineal was eventually found to be the structure responding to light in the ferret also, as pinealectomy prevented the induction of oestrus by extra hours of light per day (Herbert, 1969). Although superior cervical ganglionectomy had previously been shown to have a similar effect (Abrams, Marshall and Thompson, 1954), it was not understood at that time that this was by pineal denervation.

An extensive report on the environmental factors controlling seasonal reproduction in the ewe revealed the importance of photoperiod (Hafez, 1952). A photoperiod of 8L:16D advanced the onset of the breeding season, whereas 16L:8D accelerated the end of the breeding season. By 1970 the way in which photoperiod affected sheep was still unknown, but the demonstration of a role for the pineal in other species, prompted investigation of a role for the pineal in sheep. But pinealectomized ewes, exposed to normal seasonal photoperiod changes, did not differ from sham operated or intact controls in either the time of onset or termination of oestrous cycles, or LH levels (Roche, Karsch, Foster, Takagi and Dzuik, 1970), suggesting that the pineal was not responsible for mediating photoperiod influences. However, in rams exposed to normal and reversed seasonal changes in photoperiod, pinealectomy prevented the increase in plasma testosterone during decreasing daylength (Barrell and Lapwood, 1979). The involvement of the cranial sympathetic nervous system in mediation of photoperiod was demonstrated almost coincidentally by Lincoln (1979), who showed that superior cervical ganglionectomy (SCGX) prevented changes in plasma FSH, LH and testosterone in Soay rams exposed to alternating photoperiods of 8L:16D and 16L:8D. Long term changes in the hormonal profiles were observed though, and a similar finding was reported in pinealectomized Saxon Merino rams (Kennaway, Obst, Dunstan and Friesen, 1981). These results suggested that other environmental cues were involved or an endogenous rhythm was operative.

There is some evidence that the suprachiasmatic nuclei may generate an endogenous rhythm. In an investigation of the control of gonadotrophin secretion, Domanski, Przekop and Polkowska (1980) lesioned hypothalamic nuclei in the preoptic anterior hypothalamic area of the ewe. Animals in which bilateral lesions destroyed almost the entire SCN area showed normal oestrous cycles during the breeding season, and four of the six ewes continued to ovulate regularly in the non-breeding season. Although pineal function was not measured during this study, it is possible that the SCN lesions had prevented normal rhythmic pineal function (Moore and Klein, 1974), and consequently a normal response to increasing daylength. However, as pinealectomy alone failed to abolish seasonality (Roche *et al.*, 1970) this explanation appears too simplistic.

In other species also, the pineal gland has been shown to be involved in the translation of the photoperiodic information that controls seasonal breeding. However, as in the sheep, the pineal does not appear to be totipotent in the regulation of the annual breeding cycle; other factors, probably of endogenous origin, are also involved.

Pinealectomized ferrets showed recurrent breeding seasons for up to five years (Herbert, Stacey and Thorpe, 1978). Other environmental factors such as temperature, behavioural signals, social facilitation or the presence of a circannual rhythm, failed to explain the recurrent periods of oestrus, as they were highly asynchronous between animals and to the time of year. The most plausible explanation of their results was that of "high inertia" in the neuroendocrine system.

In the hamster, gonadal regression occurs if they experience less than 12.5 - 13 hours of light per day (Gaston and Menaker, 1967; Hoffmann, 1981). Gonadal recrudescence can then be initiated by exposure to longer photoperiods, superior cervical ganglionectomy, or pinealectomy; however gonadal recrudescence will also spontaneously occur in intact animals, after 30 weeks of exposure to short photoperiods, probably in response to endogenous factors (Reiter, 1975, 1981).

In the mare, exposure to photoperiods of increasing daylength five months early (during anoestrus) caused follicular growth, ovulation and oestrous behaviour (Sharp, Kooistra and Ginther, 1975). Similarly stallions exposed to long photoperiods (16L:8D) in winter had significantly increased testosterone levels compared to controls on ambient photoperiod (Burns *et al.*, 1982).

In mares, neither superior cervical ganglionectomy (Sharp, Vernon and Zavy, 1979), or pinealectomy (Grubaugh *et al.*, 1982), affected the onset of the first post-operative breeding season, however the second season was significantly delayed.

The annual testosterone rhythm in White-tailed deer persisted after pinealectomy, but appeared to be asynchronous with the seasonal photoperiod; this suggested that the pineal was not the generator of the annual rhythm (Snyder, Cowan, Hagen and Schanbacker, 1983).

It appears then, that the persistence of changes in reproductive status, in the absence of pineal mediated information, is a common phenomenon in seasonal breeding mammals. If, as it seems in the sheep, the SCN or another central structure(s) generates the annual breeding cycle, the pineal may function to entrain the endogenous circannual rhythm to the appropriate season, and photoperiod is the ultimate zeitgeber.

The way in which the pineal may exert its influence is discussed in the following section.

1.4 Mode of pineal influence on the reproductive axis

The classical approach to the study of endocrinology, of removing the supposed gland and challenging the system with its extracts, showed that the pineal had fulfilled one of the criteria of an endocrine gland. However, several questions still had to be answered. What was the pineal hormone? Where did it act? How did it act? This section reviews the work that attempted to answer these questions.

(i) Melatonin - the putative pineal hormone

Several compounds have been proposed as putative pineal hormones. McCord and Allen (1917) had shown that an extract of the bovine pineal could blanch the skin of frogs and tadpoles. This melanophore-lightening substance was characterized by Lerner, Case and Heinzelman (1959) as 5-methyoxy-N-acetyltryptamine (melatonin). Melatonin has since been

localized in other tissues, e.g. intestine, retina and harderian gland (Bubenik, Brown and Grota, 1977), but it is a major secretory product of the pineal (Cardinali, 1981). It was the first pineal substance to mimic the effects of photoperiod changes and pineal extracts on reproduction, and reverse the endocrine changes resulting from pinealectomy.

In the rat, constant light or pinealectomy increased ovarian weight (Fiske, 1941; Kitay, 1954). Administration of pineal extracts or melatonin decreased ovarian weight (Kitay and Altschule, 1954a; Wurtman *et al.*, 1959; Wurtman, Axelrod and Chu, 1963). Marked gonadal regression was reported in the Syrian hamster when maintained under short photoperiods (Hoffman, Hester and Townes, 1965). Melatonin injections could mimic this but, crucial for the response, was the time of day of administration. Melatonin injections given early in the light phase, or melatonin given continuously in subcutaneous implants, failed to cause gonadal regression (Reiter, 1974). But melatonin injections given shortly before the dark phase were effective in both male and female hamsters (Tamarkin, Westrom, Hamill and Goldman, 1976). The implication of this work was that putative target tissues showed a daily rhythm in sensitivity to melatonin. This will be considered subsequently in more detail.

Although melatonin was shown to be involved in mediating the effects of short photoperiods in the hamster, the question remained was it *the* pineal hormone or did it act to control the synthesis or release of another pineal compound(s), which then acted on the reproductive axis? This question has been raised by Reiter (1974) , and the failure of pinealectomized

female hamsters to become acyclic in response to daily melatonin injections that induced acyclicity in intact animals, lent support for the involvement of other pineal compounds (Tamarkin *et al.*, 1976).

The mammalian pineal gland also synthesizes the nonapeptide arginine vasotocin - AVT (Pavel, 1965), and this can mimic all of the endocrine effects of melatonin (Pavel and Petrescu, 1966; Vaughan, 1981). Pavel (1973) proposed that melatonin acts as a releasing factor for pineal AVT, as intravenous or intraventricular infusions of melatonin induced the release of AVT, and decreased pineal AVT content in cats. Furthermore, melatonin infusions were about 500 times more potent in inducing AVT release during the dark (Pavel and Goldstein, 1979). This may have explained the failure of melatonin injections during the light phase to induce gonadal regression in hamsters.

Other hormones, e.g. arginine vasopressin and oxytocin have also been proposed as pineal hormones (Vaughan, 1981), however the evidence, to be presented, that melatonin is the major pineal hormone is compelling. But where does it act?

(ii) Target tissues of melatonin

Almost no tissue in the neuroendocrine-reproductive axis appears to be free of the pineal's influence. Motta, Fraschini and Martini (1967) suggested that melatonin suppresses the secretion of LH, but that the pineal also exerts an anti-FSH effect by compounds other than melatonin (Fraschini and Martini, 1970). Melatonin injections inhibited ovulation in rats (Reiter and Sorrentino, 1971; Ying and

Greep, 1973), and this effect could be negated by the administration of LH. This suggested that melatonin did not exert its influence at the ovarian level, but on the release of pituitary gonadotrophins. Of interest, was that melatonin treatment before or after the critical period of proestrous, did not affect the incidence of ovulation.

Fraschini, Mess and Martini (1968) had shown that hypothalamic implants of melatonin decreased pituitary and plasma LH, suggesting that melatonin acted at the hypothalamic level. But the suppression of neonatal rat LH and FSH responses to luteinizing hormone-releasing hormone (LH-RH) by melatonin (Martin, McKellar and Klein, 1980), implied that the pituitary itself was the target tissue. This latter proposal was supported by the demonstration that, *in vitro*, the response of enriched rat gonadotroph cells to LH-RH was suppressed by melatonin added to the culture medium (Martin, McKeel and Sattler, 1982).

A direct effect of pineal compounds on the reproductive organs themselves could not be ruled out. Melatonin caused a further reduction in gonadal and accessory organ weights of hypophysectomized rats with already hypotrophic reproductive organs (Debeljuk, Vilchez, Schnitman, Paulucci and Feder, 1971). More recent evidence suggests that melatonin and other unknown pineal compounds act directly on the rat accessory sexual organs, by preventing the conversion of testosterone to dihydrotestosterone, and so reducing the size and function of these organs (Shirama, Furuya, Takeo, Shimizu and Maekawa, 1981, 1982).

Most evidence however, both direct and indirect, implicates the hypothalamus as the probable target (Blask, 1981). The demonstration of specific binding of melatonin in the rat hypothalamus (Niles, Wong, Mishra and Brown, 1979), and its localization in the SCN (Bubenik, Brown and Grota, 1976), suggested that melatonin acted at the hypothalamic level. In isolated, perifused, medial-basal hypothalami of rats, melatonin was shown to stimulate the release of gonadotrophin-releasing hormone. Infusions of norepinephrine, dopamine, acetylcholine or serotonin were ineffective (Kao and Weisz, 1977).

Thrice daily melatonin injections induced gonadal regression in pinealectomized male hamsters. This response was prevented by total or anterior deafferentation of the medial basal hypothalamus (Reiter *et al.*, 1981). Posterior hypothalamic cuts alone were ineffective. This suggested that melatonin may be acting on neurons that innervate the mediobasal hypothalamus anteriorly, such as from the SCN. More directly, Glass and Lynch (1981) reported that microimplants of melatonin into the preoptic, supra- and retrochiasmatic regions of the White-footed mouse caused complete gonadal regression; implants in other brain regions were ineffective. Autoradiographic analysis of brain sections revealed that tritiated melatonin only diffused to a distance of 0.2mm, indicating that melatonin was not acting at a distance from the implantation site.

(iii) Mode of influence of melatonin

Melatonin may not be the only pineal principle influencing the hypothalamic control of mammalian reproduction. However, the accumulating

evidence suggests that the temporal relationship between the circadian melatonin profile and sensitivity of target tissues, can mediate the effects of photoperiod on reproduction. It is important then, to consider the temporal nature of melatonin biosynthesis.

The synthesis of melatonin is by the hydroxylation of tryptophan to 5-hydroxytryptophan, which is decarboxylated to 5-hydroxytryptamine (serotonin). Serotonin is N-acetylated by N-acetyltransferase (NAT) to yield N-acetylserotonin, which is methylated by hydroxyindole-O-methyl-transferase to produce melatonin (Figure 1.4-1).

Stimulation of the sympathetic innervation of the pineal releases norepinephrine (NE) from the nerve terminals into the vicinity of the pinealocyte terminals, or between perikarya of the pinealocytes (Matsushima, Morisawa and Mukai, 1981). The NE binds to beta-adrenergic receptors on the pinealocyte, activating adenylate cyclase to catalyse the conversion of ATP to cAMP. This then stimulates the synthesis of NAT (Cardinali, 1981).

The activity of NAT is low during the light and increases 30 to 100-fold during the dark, but exposure to dark during the normal light phase does not increase NAT activity, as the endogenous central oscillator (SCN) is not synchronized with the imposed dark period (Binkley, Klein and Weller, 1974; Moore and Klein, 1974). Exposure to light during the dark phase will rapidly decrease NAT activity (Klein and Weller, 1972), so both exogenous (light) and endogenous (SCN) factors control NAT activity.

HIOMT activity increases gradually in constant dark and decreases in constant light, but the small daily change in activity is insufficient to account for the larger daily fluctuations in melatonin synthesis (Klein *et al.*, 1981). NAT therefore appears to be the rate-limiting enzyme in melatonin synthesis and the circadian changes in its activity are reflected in circadian changes in melatonin levels.

Circadian melatonin rhythms have been measured in pineal tissue, cerebrospinal fluid, plasma and urine of mammals. In species representing the Rodents (hamster, rat), Artiodactyla (cattle, sheep), and Primates (monkey, human), several common features of the melatonin rhythm have been described. In all groups the melatonin levels are highest during the dark phase, and in constant dark this rhythmic variation persists. Constant light, or exposure to light during the dark phase, depresses the melatonin levels (Lewy *et al.*, 1980; Lynch and Wurtman, 1981). In seasonal breeding species it is generally accepted that changes in the nocturnal melatonin rise mediate the effects of seasonal photoperiod change on reproduction. What is debated though, is which component of the nocturnal rise is the responsible hormonal signal.

Changes in the amplitude (Lincoln *et al.*, 1982), duration (Kennaway Sanford, Godfrey and Friesen, 1983) and phase (Tamarkin, Reppert and Klein, 1979) of the nocturnal melatonin rise have been described under different photoperiods, in various species. However, at the outset, it is important to note that species and breed differences may account for different reproductive responses to different components of the nocturnal rise.

As melatonin injections would only induce gonadal regression in the hamster if given just before dark (Tamarkin *et al.*, 1976) a diurnal rhythm of sensitivity to melatonin was proposed. Theoretically, the endogenous nocturnal melatonin rise changed under non-stimulatory short photoperiods and so became available during this critical sensitive period. Alternatively, under short photoperiods, the absolute levels of melatonin may have increased, and this mediated the response.

Evidence in support of an increase in absolute levels was provided by Tamarkin, Lefebvre, Hollister and Goldman (1977). They showed that melatonin injections at the onset of, or during the dark phase, to hamsters on 14L:10D, induced oestrus acyclicity or testicular regression. They suggested that the exogenous melatonin had added to the endogenous melatonin so that the circulating levels were higher, and this resulted in gonadal regression.

To determine whether the circadian profile of melatonin did differ under long and short photoperiods Tamarkin, Reppert and Klein (1979) measured pineal melatonin content in the male Syrian hamster. Under long photoperiods (14L:10D) they found a pronounced diurnal rhythm in melatonin content, dark phase values being ten-fold higher than the light phase values. Exposure to 10L:14D did not alter the amplitude or duration of the dark phase elevation, but the phasing of the melatonin rhythm relative to the time of day was altered. The onset and decline of the nocturnal rise occurred two hours earlier under 10L:14D, compared to 14L:10D. It was possible then, that the shift in the nocturnal melatonin rise made this

hormone available during a critical period. It must be noted, however, that pineal melatonin content may not truly reflect circulating melatonin levels.

As noted earlier, once-daily melatonin injections failed to induce gonadal regression in pinealectomized hamsters (Tamarkin *et al.*, 1976). However, thrice-daily injections were shown to be effective (Tamarkin, Hollister, Lefebvre and Goldman, 1977). This also supported the notion of a sensitive period, as this treatment ensured melatonin was available at the critical time. More importantly, these results showed that melatonin could not have been acting as a releasing factor for other pineal compounds, because the pineal was absent.

In sheep, two different responses of the melatonin profile to photoperiod change have been described. In the Soay ram the amplitude of the nocturnal rise increased under a long photoperiod (16L:8D) compared to 8L:16D (Lincoln and Short, 1980; Lincoln *et al.*, 1982). In Finn x Dorset/Rambouillet ewes however, the amplitude of the nocturnal rise did not differ between long and short photoperiods, although the duration coincided closely with the length of the dark phase (Kennaway, Sanford, Godfrey and Friesen, 1983).

A change in the duration of the nocturnal rise is sufficient to mimic the effect of photoperiod changes on reproduction (Kennaway, Gilmore and Seamark, 1982). Anoestrous ewes (maintained under 16L:8D), fed pellets containing melatonin 8 hours before dark, showed ovarian cyclicity earlier than the control animals. This effect was subsequently confirmed by Nett and Niswender (1982) and by Arendt, Symons, Laud and Pryde (1983).

The duration of the nocturnal melatonin profile has recently been shown to mediate the effects of inhibitory, as well as stimulatory photoperiods in the ewe. Using the LH response to oestradiol negative feedback, Bittman and Karsch (1984) demonstrated that in pinealectomized and ovariectomized ewes with oestradiol implants (OVX+E), infusions of melatonin that mimicked the endogenous nocturnal rise of long days caused a precipitous drop in LH levels; experience of an artificial short day melatonin profile caused a marked LH rise. These results suggest that the pineal, via melatonin, is involved in the measurement of daylength, rather than being either pro- or antigonadal (Bittman and Karsch, 1984).

Daily melatonin treatment before dark also brings about reproductive changes associated with short photoperiods in other species. In the White-tailed deer, oral melatonin administration advanced rutting behaviour by two months, so that it occurred in summer (Bubenik, 1983); in mink, melatonin injections delayed or inhibited the increases in plasma progesterone and prolactin associated with exposure to long photoperiods (Martinet, Allain and Meunier, 1983). However, as the endogenous melatonin profiles, or those resulting from melatonin administration were not measured in these studies, the way in which melatonin was providing hormonal information cannot be determined. It seems then, that the ability to measure putative pineal hormones is imperative.

Melatonin administration fully mimics the photoperiodic response of the ewe and hamster, as evidenced by refractoriness to

both treatments. This is further evidence that melatonin is the pineal hormone mediating the effects of photoperiod. The Soay ram becomes refractory to stimulatory decreasing photoperiod after 4 months, as determined by a decrease in testis diameter (Lincoln, 1980). As discussed earlier, hamsters become refractory to inhibitory short photoperiods within 30 weeks, and spontaneous gonadal recrudescence occurs (Reiter, 1981).

Two hypotheses of the physiology of short day refractoriness were proposed, (i) failure of the pineal to continue production of the antigonadal factor or (ii) development of insensitivity of target tissues to the antigonadal factor (Reiter, 1973). Bittman (1978) provided evidence in support of the target insensitivity hypothesis by demonstrating that a melatonin therapy regimen, known to induce gonadal regression in intact and pinealectomized hamsters, was without effect on spontaneously recrudesced hamsters. Similarly, in ewes, the breeding season was prolonged by 6 weeks by daily afternoon melatonin injections, however ovarian cyclicity eventually ceased, although the melatonin injections were maintained (Nett and Niswender, 1982). These studies show that refractoriness to short days cannot be due to a failure of the pineal to produce melatonin. Target tissues must eventually become insensitive to melatonin.

(iv) Models of reproductive seasonality

Two basic models have been proposed to explain the endocrine changes associated with reproductive seasonality. In the ewe seasonal anoestrus has been postulated to be due to either 1) reduced

sensitivity to the positive feedback response to oestrogen (Land *et al.*, 1980) or 2) increased sensitivity to the negative feedback response to oestrogen (Legan, Karsch and Foster, 1977).

Walton *et al.* (1977) suggested that hyperprolactinaemia during seasonal anoestrus was antigonadotrophic, and Kann, Martinet and Shirar, (1978) implicated hyperprolactinaemia for reducing sensitivity to feedback.

Seasonal photoperiod changes regulate the prolactin levels of various species. The highest levels occur during the period of long daylengths in the ram (Pelletier, 1973), ewe (Walton *et al.*, 1977), goat (Buttle, 1974), cattle (Karg and Schams, 1974) and deer (Schulte *et al.*, 1981). The decrease in prolactin, a response to decreasing daylength, has been suggested to be involved in the initiation of the breeding season of the ewe (Walton *et al.*, 1977).

The pineal mediates the effects of photoperiod on prolactin, but again, endogenous factors seem to be involved. In castrated male goats, superior cervical ganglionectomy performed in summer failed to prevent the decrease in plasma prolactin associated with decreasing daylength. Following ganglionectomy in winter, however, the normal rise in prolactin associated with increasing daylength was accelerated. These results suggested that a functional pineal gland was needed for the normal increase in prolactin in spring, but not for the decrease in autumn (Buttle, 1977).

The seasonal prolactin profile of White-tailed deer bucks was abolished by pinealectomy. In pinealectomized does the rhythm persisted, although the amplitude was dampened. Both sexes retained seasonality in their prolactin response to thryotropin releasing hormone, but the response was less in summer, and increased in winter, compared to control animals (Schulte *et al.*, 1981). In a longer term study on the White-tailed deer, Snyder *et al.* (1983) also reported the persistence of the seasonal prolactin rhythm in pinealectomized bucks. As discussed earlier for testosterone, this was asynchronous with the seasonal photoperiod, suggesting that the pineal was not the generator of the rhythm, but entrained the rhythm. Kennaway *et al.* (1981) also reported asynchrony between the annual prolactin rhythm and season in pinealectomized rams.

Melatonin appears to be the pineal hormone mediating the effects of photoperiod on prolactin secretion. In ewes maintained on long photoperiods, melatonin feeding that mimicked the profile of short photoperiods depressed prolactin levels (Kennaway *et al.*, 1982; Symons *et al.*, 1983).

Although the pineal regulates both seasonal reproduction and prolactin levels, and there is strong correlative evidence that the seasonal changes in prolactin are involved in reproductive seasonality, experimental manipulation of this system does not fully support this role of prolactin. A decrease in daylength reduced circulating prolactin levels of ewes, but immediate resumption of ovarian activity did not occur. Furthermore, although bromocriptine (CB154) reduced prolactin concentrations to undetectable levels in anoestrous

ewes, the onset of oestrus was only marginally advanced (Walton *et al.*, 1980). Land *et al.* (1980) also questioned the importance of hyperprolactinaemia for anoestrus, as again, treatment of anoestrous ewes with bromocriptine reduced prolactin levels dramatically, but there was no increase in the number of ewes ovulating.

The second model to account for regulation of seasonality proposes that the pineal alters the sensitivity of the hypothalamus to negative steroid feedback. Although the evidence for a seasonal change in hypothalamic sensitivity has been compelling in the sheep and hamster, more recent evidence has revealed anomalies in this model also.

Legan, Karsch and Foster (1977) demonstrated that OVX+E ewes showed striking seasonal changes in circulating LH. It was undetectable during the normal period of anoestrus, but greatly elevated during the normal breeding season. As discussed previously, a role of the pineal in this change was suggested by Bittmann and Karsch (1984). In pinealectomized, OVX+E ewes, infusions of melatonin that mimicked the endogenous nocturnal rise of long photoperiods caused a dramatic drop in LH levels; experience of an artificial short day melatonin profile caused a marked LH rise. However, the role of the pineal and steroid sensitivity in seasonal reproduction has been questioned by Kennaway, Dunstan, Gilmore and Seamark (1983). In their study, pinealectomized ewes showed normal reproductive seasonality, yet a seasonal rhythm in oestradiol negative feedback on LH was absent. They concluded that seasonal breeding could not be attributed to seasonal changes in steroid sensitivity. Although the pineal entrains the breeding cycle,

it does this by acting directly on the neuroendocrine axis. Negative steroid feedback is only a reflection of pineal hormone action (Kennaway Dunstan, Gilmore and Seamark, 1983).

Early work in the hamster had also suggested that the pineal controlled seasonal breeding by altering sensitivity to negative steroid feedback. Hamsters showed an increase in serum FSH and LH after orchidectomy. These were reduced in a dose-dependent manner by testosterone implants. The dose required to suppress these gonadotrophin levels was less under non-stimulatory (6L:18D) compared to stimulatory photoperiods (14L:10D), and this effect was prevented by pinealectomy (Turek, 1979).

There is evidence, however, that the photoperiod response of the hamster is steroid independent. The plasma FSH and LH concentrations of castrated hamsters increased during exposure to long photoperiods, and decreased under short photoperiods (Simpson, Follett and Ellis, 1982). Both the pineal gland and SCN mediate this steroid-independent decrease in FSH under short photoperiods, as lesioning the SCN, or removal of the pineal at the time of castration abolishes the response (Turek, Losee-Olson and Ellis, 1983).

In summary, it is obvious that many of the previously well-accepted views of the pineal's role in seasonal reproduction may need to be reassessed. However this review has shown that several general principles about the mammalian pineal can be postulated.

1. The pineal has physiological importance in reproduction: it is not vestigial.
2. It can be regarded as an endocrine gland, having at least one biologically active compound.
3. Melatonin is a major pineal hormone.
4. It is changes in the circadian melatonin profile that mediate reproductive responses to photoperiod.
5. The pineal can be both 'pro-' and 'antigonadal', either of which being determined by the normal response of the animal to the prevailing photoperiod environment.

The following section will examine whether these general principles also apply to what is already known of the pineal and reproduction in marsupials.

1.5 The Marsupials

Relatively little direct work has been done on the role of photoperiod and the pineal in marsupial reproduction. Most of the literature to date on the marsupial pineal concerns anatomical studies. Although this is not the main subject of this section, these studies need to be reviewed to highlight similarities to, or alternatively any major departures from, the 'typical' mammalian pineal.

(i) Marsupial pineal anatomy

Fortuitously, anatomical studies of the marsupial pineal had been done on representative groups, and from what had been reported at that time Quay and Baker (1965) commented on the "diversity of

pineal structure and composition within the marsupials possibly greater than that occurring in eutherian mammals".

All descriptions of the marsupial pineal show that it is attached to the posterior roof of the diencephalon, anterior to the superior colliculi. The elongated pineal stalk described in some rodents, appears to be absent in the Marsupiala. The pineals of the red and grey kangaroo (*Macropus rufus* and *M. giganteus*) are macroscopically distinct (Quay and Baker, 1965). In the red kangaroo it is symmetrically bilobate; in the grey kangaroo, it is rounded distally. The bilobate form has been described in one species of bandicoot, *Perameles gunnii* but not another, *P. nasuta* (c.f. Quay and Baker, 1965). An apical notch described in the wombat (species unknown) may be suggestive of the bilobate condition (c.f. Quay and Baker, 1965).

A more recent cytological survey of the pineal region of 11 species of marsupials, representing the families Dasyuridae, Petauridae, Phalangeridae, Phascolarctidae and Macropodidae, by Kenny and Scheelings (1979) also revealed a very varied morphology within this group. Although being a well defined structure in all species examined, it varied greatly between groups in size, and in the possession and type of pineal recess of the third ventricle. From histological sections, the pineals of the Macropodidae and Phascolarctidae were relatively larger than in the remaining groups, while among the Dasyuridae the pineal of *Dasyuroides byrnei* was larger than that of *Sminthopsis crassicaudata* and *Antechinomys laniger*.

A true pineal recess was described in some species of each family except the Phalangeridae, of which only one species, *Trichosurus vulpecula*, was examined (Kenny and Scheelings, 1979). A similar, well-defined pineal recess is also described in the short-nosed bandicoot *Isoodon macrourus* - Peramelidae (Harsthorn and Bryden, 1982), and the potoroo *Potorous tridactylus apicalis* - Macropodidae (Bradley, 1973). In *D. byrnei* and *A. laniger*, and possibly *Schoinobates volans* (Petauridae), a nerve attached to the superior surface of the pineal may be homologous to the *nervus conarii* of various eutherian mammals (Kenny and Scheelings, 1979). No *nervus conarii* was evident in the potoroo (Bradley, 1973).

Only one type of pinealocyte was described in the potoroo and its distribution appeared to be random (Bradley, 1973). However a prominent basal to distal zonation of pineal parenchymal cells was described in both red and grey kangaroo (Quay, 1966).

Apart from pinealocytes and glial cells, there is evidence that rudimentary photoreceptor cells also contribute to the formation of the pineal gland in the adult brushtail possum (*Trichosurus vulpecula*). Scattered among glial cells in the transitional zone adjacent to the subcommissural organ, are cells characterized by ciliated inner segments, the ellipsoides of which protrude into the pineal lumen (Samarasinghe, Wong and Webster, 1982). These cells are similar to the pineal photoreceptor cells of submammalian vertebrates that also possess a modified cilium or flagellum which extends into the epiphyseal or parapineal lumen. These cells, in turn, are morphologically similar to retinal rods and cones (Wurtman, Axelrod and Kelly, 1968).

(ii) Marsupial pineal physiology

From what information is available, the physiology of the marsupial pineal appears to be similar to that of eutherians. Melatonin and 5-hydroxyindole-3-acetic acid levels of red and grey kangaroo pineals collected at night, were similar to those recorded for rats in the dark. Serotonin levels, however, were lower (Quay and Baker, 1965). Hydroxyindole-O-methyltransferase and monoamine oxidase activity was detected in the pineals of tammars (*M. eugenii*) also collected at night (Kennaway and Seamark, 1976). In both studies, however, daytime levels were not reported. The first demonstration of a light/dark change in marsupial pineal function was provided by Renfree *et al.* (1981), who showed midnight plasma melatonin levels to be 3 to 5 times greater than those measured at midday.

An anatomical link between the superior cervical ganglia and pineal in marsupials was demonstrated by degeneration studies in the potoroo (Bradley, 1973), and abolition of the nocturnal melatonin rise by superior cervical ganglionectomy showed this to be a functional link in the tammar (Renfree *et al.*, 1981). The remaining elements of the central neural pathway controlling pineal function in marsupials are not known. However a retinohypothalamic projection to the SCN, implicated in the regulation of the rat pineal (Moore and Klein, 1974), has been described in *Didelphis virginiana* (Moore, 1973), *D. marsupialis aurita* (Cavalcante *et al.*, 1975), *Pseudocheirus peregrinus*, (Pearson *et al.*, 1976), *Marmosa mitis* (Royce *et al.*, 1976), *Trichosurus vulpecula* (Sanderson *et al.*, 1978), *Sarcophilus harrisii* (Sanderson *et al.*, 1979), *Lasiorhinus latifrons* (Sanderson *et al.*, 1981) and the tammar (Wye-Dvorak, 1984).

(iii) Photoperiod effects on reproduction

Before considering the evidence implicating photoperiod in regulation of marsupial reproduction, an apparent anomaly in their seasonality needs to be clarified. Some seasonally breeding marsupials, e.g. macropods, give birth in summer. In contrast, comparable eutherians give birth in spring, a period most conducive to survival of their young. In marsupials, however, the major maternal investment is in lactation, which may range, in different species, from 50 - 275 days, and not in gestation, as it is in eutherians. Hence, it is emergence from the pouch that occurs during spring, and so the proximal factor(s) leading to conception must operate at a time that caters for both gestation and pouch life (Sharman, Calaby and Poole, 1966; Tyndale-Biscoe, 1980).

In marsupials that have a short gestation and pouch life, breeding may occur quickly in response to favourable conditions such as increasing temperature, or the availability of protein rich food, but photoperiod may also be involved (Tyndale-Biscoe, 1980). However, in seasonal breeding marsupials with a longer pouch life, breeding may have to occur up to nine months before the favourable season. Photoperiod appears to be the proximal environmental factor controlling breeding in these species.

In the Virginia opossum (*Didelphis virginiana*), the breeding season begins in late January; increasing daylength after the winter solstice (in December) may be involved, because extending the length of daylight after the autumnal equinox (in September) initiated premature

breeding (Farris, 1957). A similar effect was shown in the Fat-tailed dunnart, *Sminthopsis crassicaudata*. Gradually increasing daylength from 12 to 15 hours after the autumnal equinox (in March) initiated the start of the oestrous period 9 weeks early. The control group came into oestrus soon after the winter solstice and the change to increasing daylength. However, sexual maturation of the males was not advanced by the artificial photoregimen (Godfrey, 1969).

In a subsequent study of *S. crassicaudata* exposure to 8L:16D abolished oestrous cycles, but these then resumed 20-30 days after a change to 16L:8D (Smith *et al.*, 1978). Of particular interest was that very few litters were born to animals maintained on long photoperiods for more than six months. It seems that these marsupials become refractory to stimulatory photoperiods; as discussed previously, this phenomenon occurs in the hamster (Reiter, 1981) and sheep (Lincoln, 1980).

In the planigale, *Planigale tenuirostris*, constant photoperiod (12L:12D) inhibited sperm production and copulatory behaviour (Davey and Croft, 1982), so a change in photoperiod may be important for this species also.

As all the remaining literature on the effects of photoperiod are concerned with the tammar, these will be included in the following section.

1.6 The reproductive biology of the tammar, *Macropus eugenii*

(i) Introduction

The tammar is a small wallaby (3-6kg) that is restricted to some islands and isolated mainland regions of southern Australia. The female is a strict seasonal breeder, but the male is capable of breeding at all times of the year.

The following sections will outline the general features of the tammar's reproductive biology, and the evidence implicating a role for photoperiod and the pineal in seasonal reproduction of this species. Finally, why and how this project was formulated will be discussed.

(ii) The pregnant and oestrous cycles

Between 8 and 16 hours after birth the female enters oestrus. If she is maintained with sexually mature males this can usually be detected by the presence of a copulatory plug in the urogenital sinus. An LH surge occurs 0-8 hours after the onset of oestrus, resulting in ovulation 24-40 hours later (Sutherland, Evans and Tyndale-Biscoe, 1980). The newly formed corpus luteum (CL) becomes quiescent in response to the sucking stimulus of the newborn. If conception results from the post-partum oestrus, the embryo will develop to a unilaminar blastocyst of 80-90 cells (Berger, 1966), but enters diapause while the CL is quiescent.

Removal of the pouch young (RPY) before June, in the southern hemisphere, reactivates the CL. This is characterized by hyperplasia and hypertrophy of the luteal cells (Renfree and Tyndale-Biscoe, 1973; Renfree, Green and Young, 1979). A pulse of plasma progesterone of luteal origin (from 200-450pg/ml) can be detected 5-8 days after RPY, and lasts for 1-2 days. From Day 10, the levels gradually increase again (to 500pg/ml), and remain high until parturition or oestrus (Hinds and Tyndale-Biscoe, 1982a). If present the blastocyst also reactivates, and birth occurs 26-27 days after RPY followed by a post-partum oestrus (Merchant, 1979). In non-pregnant tammars the progesterone profile is similar and oestrus alone occurs 28-31 days after RPY (Hinds and Tyndale-Biscoe, 1982a; Merchant, 1979).

(iii) The annual reproductive cycle

Eighty per cent of the female population give birth in the summer months of January and February. A tammar born at this time is usually weaned in the spring months of September or October. After weaning, the blastocyst and/or CL that were formed after birth in January-February remain quiescent, and are reactivated after the summer solstice in December. Birth and/or oestrus occurs in January or February and a new CL and blastocyst are formed (Figure 1.6-1).

As stated previously, if the pouch young is experimentally removed (RPY) or dies before June, the CL and blastocyst reactivate, and birth/oestrus occur about 26-28 days later. From July to December, however, reactivation does not occur in response to removal or weaning of the pouch young (Figure 1.6-1). Furthermore, young

females reaching sexual maturity in this period will come into oestrus (Andrewartha and Barker, 1969), but the CL then enters quiescence (Tyndale-Biscoe and Hawkins, 1977).

There are two factors then, that initiate and maintain quiescence of the CL and blastocyst in the tammar. From January to June the sucking stimulus of the pouch young is involved, as RPY or mammary gland denervation (Renfree, 1979) reactivates the CL. This period is therefore called lactational quiescence. From July to December environmental factors are thought to be involved, so this period has been termed seasonal quiescence (Figure 1.6-1).

The pituitary gland mediates both of the lactational and seasonal influences on quiescence as hypophysectomy at either time reactivates the corpus luteum (Hearn, 1973, 1974). Prolactin appears to be the pituitary hormone suppressing the CL. Prolactin injections after RPY in lactational quiescence, or after hypophysectomy in seasonal quiescence, delay reactivation for the duration of the treatment (Tyndale-Biscoe and Hawkins, 1977). Prolactin may exert its influence directly on the CL, as prolactin receptors are in greater concentration on the CL than any other tissue, including the lactating mammary gland (Sernia and Tyndale-Biscoe, 1979; Stewart and Tyndale-Biscoe, 1982). The rate of progesterone synthesis of the CL *in vitro* is unaffected by prolactin so prolactin may not inhibit steroidogenesis *per se*, but achieves this by preventing hyperplasia and hypertrophy of the luteal cells (Hinds, Evans and Tyndale-Biscoe, 1983).

Seasonal changes in prolactin are reported in the tammar. During lactational quiescence plasma prolactin concentrations ranged from 26.0 to 37.6ng/ml. In non-lactating females the levels during seasonal quiescence increased from 49.8 to 60.3ng/ml. At the summer solstice (December 22) the levels were low again (Tyndale-Biscoe and Hinds, 1984). In a separate study, the levels in lactating females were even higher (>100ng/ml) in seasonal quiescence (Hinds and Tyndale-Biscoe, 1982b), so both seasonal and lactational influences appear to be additive during seasonal quiescence (Tyndale-Biscoe and Hinds, 1984).

Photoperiod may control the seasonal prolactin rhythm as 1) the levels are low during the period of decreasing daylength (lactational quiescence), and elevated during increasing daylength (seasonal quiescence), and 2) artificial photoregimens affect prolactin levels. Prolactin levels were high (>40ng/ml) in animals maintained on 15L:9D in seasonal quiescence, but after an abrupt change to 12L:12D the levels had declined to <30ng/ml within 14 days (Hinds and den Ottolander, 1983).

Although prolactin is known to be involved in maintaining quiescence, it is not clear whether the seasonal prolactin changes alone control the annual breeding cycle. In lactational quiescence a single injection of bromocriptine (5mg/kg) caused immediate reactivation of the CL in 14 of 15 treated animals. In seasonal quiescence however, only 1 of 25 animals responded to the same treatment. This cannot have been due to the failure of bromocriptine to depress prolactin in seasonal quiescence as bromocriptine injections failed to depress prolactin at either time of the year, and animals

treated at the summer solstice (December), when levels were low again, did not respond to this treatment, but did so two months later when levels were similar (Tyndale-Biscoe, and Hinds, 1984).

Although prolactin is involved, the endocrine control of lactational and seasonal quiescence is clearly different, and another factor, perhaps of pineal origin, is also involved (Tyndale-Biscoe and Hinds, 1984).

(iv) Evidence for the involvement of photoperiod and the pineal

There are various pieces of evidence, both direct and indirect, that implicate photoperiod as the environmental factor controlling the annual reproductive cycle of the tammar, and that this may be mediated by the pineal.

Firstly the annual photoperiod change of southern Australia is from about 15 hours of light/day at the summer solstice (December 22) to about 9 hours at the winter solstice (June 22). The dates of the first births of the breeding season range from January 14 to February 16, and the mean dates of several seasons are January 28-31 (Tyndale-Biscoe, *pers. comm.*). As the gestation period is 26-27 days, blastocyst reactivation must occur shortly after the summer solstice (December 22). Tammars taken to the northern hemisphere reversed their breeding season to also give birth one month after the summer solstice, which occurs there in June (Berger, 1970).

The photoperiod signal that induces reactivation appears to be the change to decreasing daylength after the summer solstice, and not long daylength itself. Exposing tammars to 15L:9D in seasonal quiescence (for 40 days from August) did not induce reactivation, but after a change to 12L:12D births occurred 29-36 days later (Sadleir and Tyndale-Biscoe, 1977). In a previous study, however, tammars exposed to an advanced photoregimen of summer solsticial photoperiod 5 weeks early (in November), and then decreasing daylength, did not respond to this treatment but they gave birth at the normal time in February (Hearn, 1972). Together these results suggest that for a decrease in daylength to be effective the animals may have to experience long daylength for a prerequisite period. In Hearn's study, the tammars may not have been sufficiently 'primed' for the decrease in daylength to be effective.

Although photoperiod change is a powerful stimulus, it is not necessary for blastocyst reactivation to occur. In Hearn's study, the time of births was not advanced by the advanced solsticial photoperiod, and births occurred at the normal time in February. In the study of Sadleir and Tyndale-Biscoe, a separate group of animals were maintained on 15L:9D from the vernal equinox (September) and births occurred in December and January at the same time as the controls. It is clear that factors other than photoperiod must be capable of inducing reactivation at the start of the breeding season. As will be shown, this had a significant influence on the direction of the present study. However, as photoperiod is probably the predominant factor for

the tammar, the question remains whether the pineal mediates this information, as it does in other mammalian species.

The first indication that the pineal may be involved in reproduction of the tammar was from the work of Kennaway and Seamark (1976). The pineal glands of tammars were obtained each night around the summer solstice from December 21 to 29, and the total soluble protein content, and HIOMT and MAO activity of the organs measured. HIOMT activity was lowest around the time when blastocyst reactivation occurred in the majority of animals. However, as these animals were spotlighted before being shot (Tyndale-Biscoe, *pers. comm.*) and HIOMT activity is decreased by light (Klein *et al.*, 1981), the significance of this finding is dubious.

Renfree *et al.* (1981) demonstrated that the superior cervical ganglia were necessary for the manifestation of seasonal quiescence. Ganglionectomy performed from March-May (in lactational quiescence) abolished seasonal quiescence in all of the 8 treated animals; removal or loss of the pouch young during seasonal quiescence (in July-November) reactivated the CL and blastocyst, and births occurred 27-28 days later, followed by oestrus. None of the sham operated or intact controls responded to this treatment; they remained in seasonal quiescence and gave birth at the normal time in February. Ganglionectomy abolished the nocturnal melatonin rise, so if, as in other species, the effects of pinealectomy and ganglionectomy on seasonality of the tammar are the same, this study showed that the pineal was necessary to initiate and maintain seasonal quiescence.

1.7 The scope and aims of this thesis

The female tammar was chosen for this study as its reproductive biology was better known than in any other marsupial, and it was the only marsupial species in which a possible role of the pineal in reproduction had been demonstrated. Males were not used as they are not truly seasonal (Catling and Sutherland, 1980). In order to elucidate the role of the pineal in reproduction of the tammar, my study began with three basic objectives; to examine

(i) the effect of pinealectomy on seasonality

(ii) the normal melatonin profiles throughout the annual breeding cycle and under a stimulatory artificial photoregimen, and

(iii) the effect of melatonin administration on seasonal quiescence.

To these ends the first task was to develop a reliable procedure for pinealectomy. This was accomplished and I am greatly indebted to Professor R.F. Mark, Dr. C.H. Tyndale-Biscoe and Dr. L.A. Hinds for theoretical and practical assistance in both the anaesthesia and surgery itself. Pinealectomy was considered preferable to ganglionectomy, although in the course of the study it became obvious that a comparison of these treatments was needed, and so a procedure for ganglionectomy was also developed. Chapters 3 and 4 outline these procedures, and in Chapter 6 the effects of each procedure at different times of the year are considered.

In order to monitor plasma melatonin levels, an established radioimmunoassay was used, and Chapter 5 outlines modifications to the assay and its validation for use in the tammar. The ability to measure melatonin had a two-fold purpose. Firstly, changes in the circadian plasma melatonin profile were used to assess the integrity of the surgical procedures. Secondly, if melatonin was the pineal hormone involved in seasonality of the tammar, it was necessary to know what the seasonal changes in the circadian profile were, and how these were affected by photoperiod change and melatonin treatment. Without this knowledge it would not be possible to explain how melatonin caused any reproductive effects. In Chapter 7 evidence for a correlation between changes in the circadian melatonin profile and reproductive status was sought, and this information was used to test the effects of melatonin administration. Chapter 8 is a recapitulation of the preceding Chapters, and discusses how the pineal is involved in the annual reproductive cycle of the tammar.

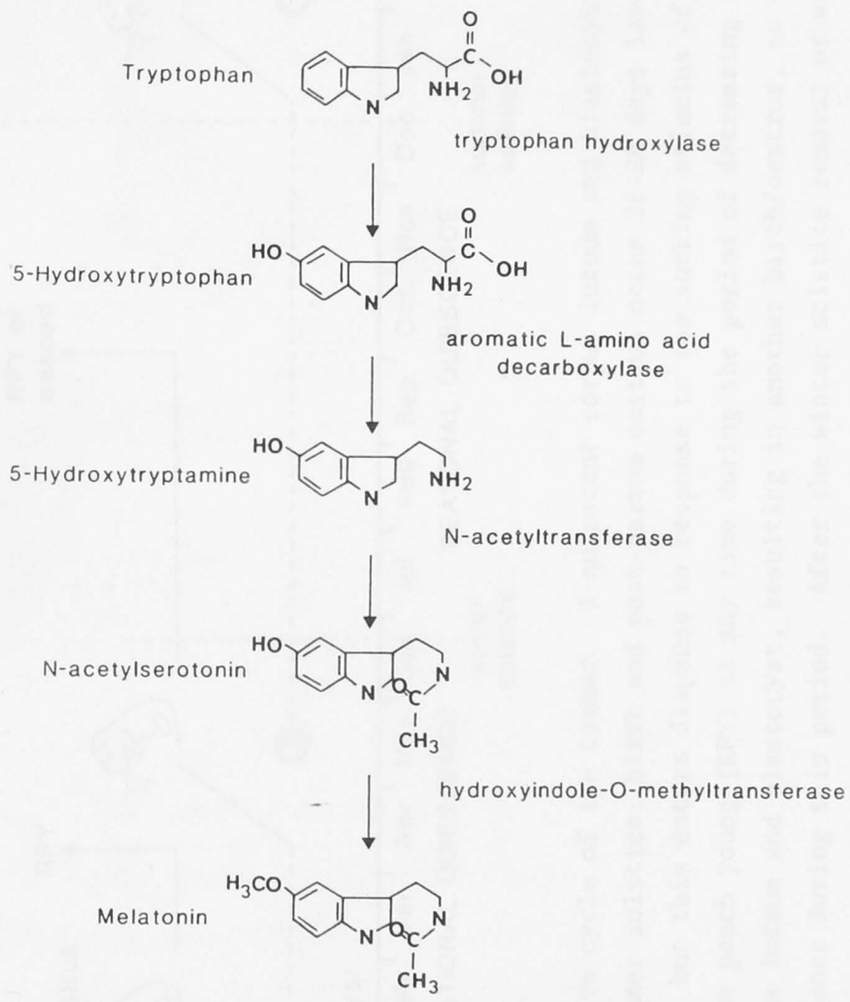


Figure 1.4-1: The biosynthesis of Melatonin from Tryptophan (redrawn from Quay, 1974, p.140).

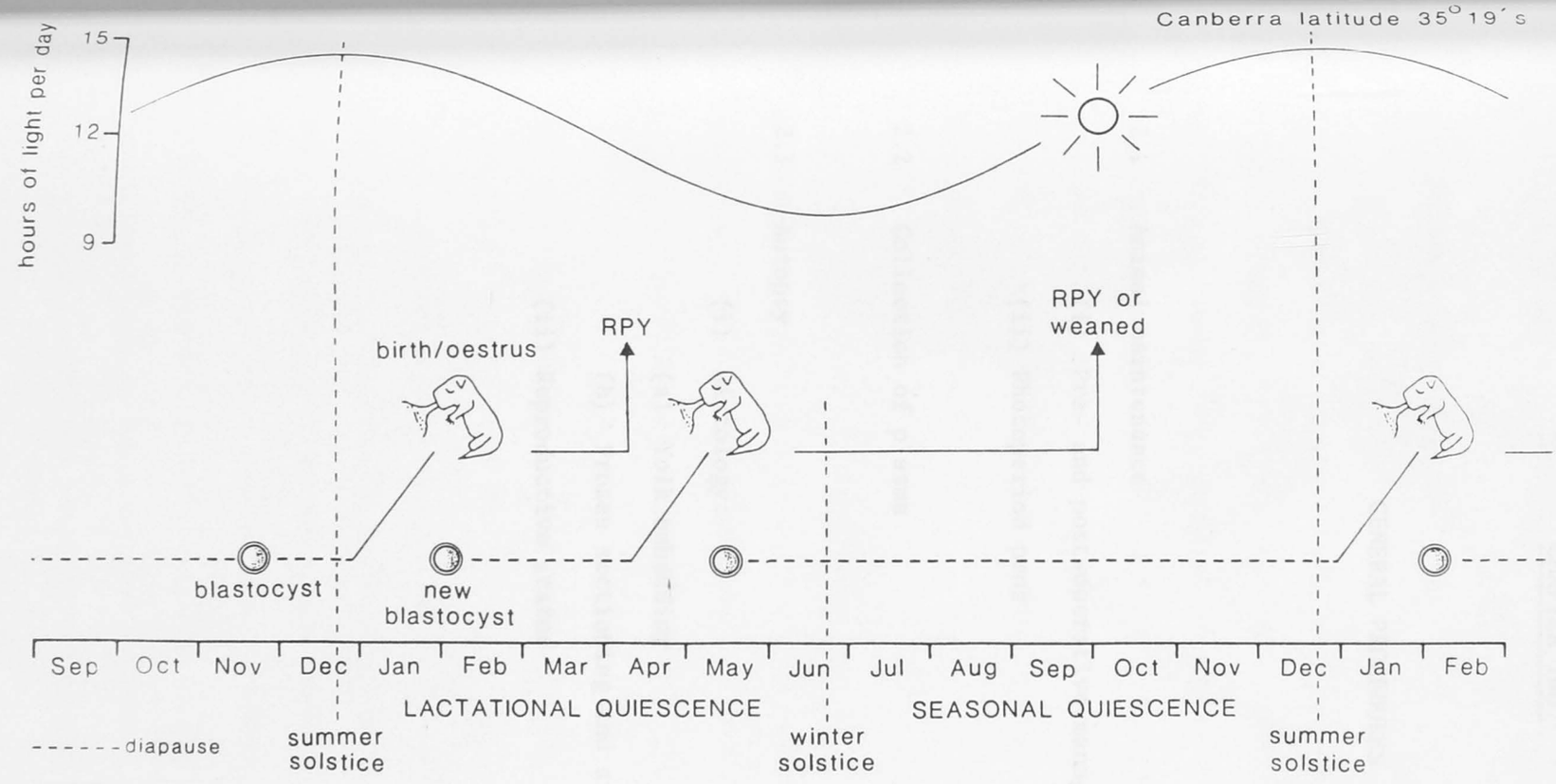


Figure 1.6-1: The annual reproductive cycle of the tammar. A quiescent corpus luteum and blastocyst reactivate shortly after the summer solstice; birth and post-partum oestrus occur 26-28 days later. A new blastocyst is formed, but this enters diapause in response to the sucking stimulus of the pouch young. Removal of the pouch young (RPY) at any time during the period of decreasing daylength reactivates the corpus luteum and blastocyst, resulting in another birth/oestrus, so the females are in lactational quiescence during this period. After the winter solstice removal or weaning of the pouch young does not reactivate the corpus luteum and blastocyst. This seasonal quiescence continues during the period of increasing daylength and is terminated at the start of the new breeding season after the summer solstice.

CHAPTER TWO

GENERAL PROCEDURES

2.1 Animal maintenance

(i) Pre- and post-operative management pens

(ii) Photoperiod pens

2.2 Collection of plasma

2.3 Autopsy

(i) Histology:

(a) Yolk embedding

(b) Frozen sectioning and staining

(ii) Reproductive status

GENERAL PROCEDURES

2.1 Animal maintenance

Tammars used in this study were obtained from colonies maintained at the C.S.I.R.O. Division of Wildlife and Rangelands Research, Canberra, and the Department of Behavioural Biology, R.S.B.S., Australian National University, Canberra, A.C.T. The C.S.I.R.O. colony is derived from an original stock brought from Kangaroo Island, South Australia in 1973-74; the ANU colony is also derived from Kangaroo Island tammars and was established in 1980.

At both Institutions tammars were kept in open yards with sexually mature males, provided with various types of shelters and allowed free access to lucerne chaff/oat pellets and water. When required tammars were transferred to the following pens:

(i) Pre- and post-operative management pens

Prior to and following surgery the tammars were maintained in holding pens at C.S.I.R.O. The dimensions of each pen were 1.25m x 1.6m which was large enough to hold three animals. Shelters constructed of hessian bags and straw bedding were provided. The room containing the pens was provided with artificial heating (20 - 24^{o}C) during cold periods and received natural environmental lighting. Food (pellets supplemented with cabbage leaves) and water were available *ad libitum* until 16 hours before surgery.

(ii) Photoperiod pens

Three types of photoperiod rooms were used during the study. At C.S.I.R.O. these were either:

(a) those previously described (Sadleir and Tyndale-Biscoe, 1977) being 4.4m x 1.2m and able to hold 5 animals each. Two 40W 'Daylight' flourescent tubes (Osram) suspended 1.8m above the floor provided illumination. Wood shavings were used as litter and hessian shelters were provided. Temperature was ambient.

(b) 1.8m x 1.85m with identical lights suspended 3.2m above the floor. One 15W red pilot lamp provided low illumination during dark periods. Straw litter and hessian shelters were provided and temperature was maintained at 24^{o}C. Each of these pens held 4 animals.

(c) At A.N.U. tammars were maintained individually in primate cages 0.9m x 0.73m. Illumination in the room during the light phase was provided by four 40W 'Daylight' flourescent tubes which provided up to 1250 footlamberts (one footlambert = 3.42626 candles/m^{2}), measured with a Minolta Autospot 1^{o} photometer). During the dark phase a 15W red pilot lamp provided low illumination. A hessian floor and shelter were provided in each cage. Temperature was maintained at 24^{o}C.

Whilst maintained in the photoperiod pens, the animals were fed the lucerne chaff/oat pellets and water, supplemented with cabbage leaves.

2.2 Collection of plasma

Two methods of blood sampling were used depending on the experimental protocol. For acute sampling regimes blood from the lateral tail vein was drawn into heparinized plastic disposable syringes using 19 or 20 gauge needles. This method was found to be both stressful and unreliable for frequent sampling over a long period, as the injection site became obviously sore and swollen, so a chronic indwelling catheter was used.

A 20 gauge 'Surflow' I.V. catheter (Terumo) was implanted into the lateral tail vein close to the rump and strapped securely with gauze and masking tape. The catheter was flushed daily with 0.2ml of heparinized saline (250 i.u./ml) and blood samples were obtainable for up to 6 days from the same catheter.

The blood was kept on ice until centrifuged and plasma stored in 2.5ml polythylene vials (Kartell) at -15^{o}C until assayed. During chronic bleeding regimes the blood cells were resuspended in a buffered sodium citrate solution, using an aspetic technique, and returned to the animal at the subsequent bleed to maintain haematocrit. Tammars subjected to frequent blood sampling and blood cell replacement were given 1ml Aquacaine L/A antibiotic suspension intramuscularly as a prophylactic measure. No animal was found to develop obvious infection as a result of these procedures.

2.3 Autopsy

(i) Histology

To confirm the integrity of the pinealectomy procedure by histological examination the first group of tammars that were pinealectomized or sham-operated in October 1981 were sacrificed by barbiturate anaesthesia (Surital, Parke-Davis) and the brain perfused with 0.9% saline and 10% formol saline.

The thorax was opened and, after clamping the descending aorta, the left ventricle was pierced with a 20 gauge needle connected to a controlled flow intravenous giving set, and the 0.9% saline was gravity fed into the ventricle. Having confirmed correct placement of the needle by swelling of the right auricle, the auricle was pierced and when the venous return was seen to be clear one litre of formol saline was infused. Fixation of the head was indicated by stiffening of the neck, ears and lips. The head was then amputated, the skull opened using rongeurs, and placed in formol saline until the brain had hardened. The brains were blocked to include all of the epithalamic region. The block was then placed in a 30% sucrose/formol saline solution which prevented tissue damage by the formation of ice crystals during freezing. Adequate impregnation of the sucrose solution was assured after the block no longer floated.

To prevent loss of pineal tissue during sectioning the block was then embedded in egg yolk.

a) Yolk embedding. The block was oriented in cardboard boats 5 x 4 x 4cms and covered with homogenized hen's egg yolk, ensuring no air pockets developed. The boat was immersed in formol saline. After 24-48 hours the hardened yolk was removed from the boat and trimmed for frozen sectioning.

b) Frozen sectioning and staining. The yolk block was oriented on the stage of a freezing microtome (American Optical) using O.C.T. Compound (Tissue-TEK II) and frozen with compressed carbon dioxide gas. Sagittal serial sections (40μm) were cut, floated in distilled water and mounted on gelatin-coated microscope slides. After drying at room temperature the sections were defatted and stained with cresyl violet as follows:

Defatting

1) 50% alcohol for 2 minutes
2) 70% alcohol for 2 minutes
3) 80% alcohol for 2 minutes
4) 95% alcohol for 2 minutes
5) 100% alcohol for 2 minutes
6) Fresh 100% alcohol for 2 minutes
7) Xylene for 2 minutes
8) Fresh Xylene for 2 minutes

Rehydration:

1) 100% alcohol for 2 minutes
2) 95% alcohol for 2 minutes
3) 80% alcohol for 2 minutes
4) 70% alcohol for 2 minutes
5) 50% alcohol for 2 minutes
6) Distilled water for 2 minutes
7) Fresh Distilled water for 2 minutes

Staining and Dehydration

1) Cresyl violet (0.5%, pH 3.4) for 3-60 seconds
2) 70% alcohol for 5 seconds
3) 80% alcohol for 5 seconds
4) 95% alcohol for 2 minutes
5) 100% alcohol for 2 minutes
6) Fresh 100% alcohol for 2 minutes
7) Xylene for 2 minutes
8) Eukitt or DePex mounting medium and coverslip.

The sections were examined for remnants of pineal tissue in the pinealectomized animals or a patent pineal in the sham operated animals.

(ii) Reproductive status

The urogenital system was dissected out to record the following:

- ovarian weight
- uterine weights and pregnancy
- corpora lutea weight and location
- corpora albicantia

To recover ova or embryos from the uteri the utero-tubal junction was cut and a blunt 18 gauge needle inserted through the cervix. The uterus was flushed with 2.5ml 0.9% saline and repeated if necessary. The irrigant was examined under a binocular microscope and the presence and condition of ova or conceptus noted.

CHAPTER 3

DEVELOPMENT OF A PROCEDURE FOR PINEALECTOMY

DEVELOPMENT OF A PROCEDURE FOR PINEALECTOMY

3.1 Surgical approach

In order to define the approach to the pineal that would cause the least trauma to other tissues and organs of the head, a wallaby was sacrificed by overdose of intravenous barbiturate (Nembutal-Abbott), and the upper trunk and head perfused with normal saline and 10% formol saline as described in Chapter 2.3. After removing the brain, still enshrouded by the meninges, the locations of the pineal and other epithalamic structures were determined.

The lack of a corpus callosum in the tammar allowed a direct approach to the pineal from the top of the skull without division of nerve tracts. The cerebral hemisphere was able to be displaced laterally, and the falx cerebri acted to prevent the opposite hemisphere from shifting also and obstructing the approach, as has been reported during earlier attempts at pinealectomy of the tammar (Tyndale-Biscoe, *pers. comm.*). No major blood vessels obstructed the approach and the inferior sagittal sinus could be displaced without being ruptured.

Aspiration of the pineal was considered preferable to extirpation using forceps as aspiration would allow for easier removal of any pineal tissue remnants.

3.2 Anaesthesia and surgical preparation

After fasting overnight the animal was weighed and anaesthesia induced and maintained by infusion of a 4% solution of sodium thiamylal

(Surital-Parke-Davis) into the lateral tail vein, followed by 0.2ml of Atropine Sulphate (0.6mg/ml-Glaxo). After removing the pouch young the scalp region was shaved and scrubbed with a 1% solution of Cetavlon and the animal placed on a warming pad (37^{o}C). The head was secured to a platform mounted on a ball joint using a mouth bar and masking tape (Fig. 3.2-1), and draped with a sterile operating cloth. The ball joint allowed fine adjustments of head orientation that were necessary during surgery. The following procedures were performed using a binocular operating microscope (Carl Zeiss).

3.3 Surgical procedure

A midline scalp incision was made extending from 5mm rostral of the coronal suture to the occipital protuberance using electrocautery (Surgistat-Valleylab, Colorado). The periosteum between the sagittal suture and left parietal ridge was deflected and the aponeurosis of the temporalis muscle freed. The scalp and muscle were retracted using towel forceps. A 14mm x 7mm plate was cut through the parietal bone using a Torx drill (J. Morita Corp. Japan) fitted with a round dental burr to the underlying dura mater, 2mm caudal to the coronal suture and 1mm lateral to the sagittal suture (Figure 3.3-1). The bone plate was lifted at the lateral furrow cracking any adherent bone, and placed in sterile 0.9% saline. The exposed dura mater was irrigated with 0.9% saline and debris aspirated.

The dura was then pierced and lifted with the bent tip of a 20 gauge hypodermic needle and cut with iridectomy scissors. A 2-3mm

margin of the dura at the rostral, caudal and ipsilateral edges provided protection to the cerebral cortex during hemispheric displacement and support for the replaced bone plate. The dural flap was deflected over the sagittal suture and kept moist with 0.9% saline-soaked gelatin sponge (Gelfoam-Upjohn). The superior sagittal sinus was not obvious and usually did not rupture during drilling or hemispheric displacement. Blood vessels were found extending mediolaterally in the pia mater which could usually be coagulated and cut using electrocautery.

Using a small round ended spatula, and aspiration of cerebrospinal fluid through a smooth tipped 18 gauge needle, the left hemisphere was displaced to expose the interhemispheric fissure and falx cerebri down to the inferior sagittal sinus. Adhesion of the sinus to the left hemisphere was gently freed with the aspirator, and the choroid plexus displaced to expose the caudal roof of the diencephalon and the hippocampal and habenular commissures.

The pineal gland was found connected, and lying caudal, to the habenular commissure (Fig. 3.3-2 and 3.3-3). A mass of vascular tissue often obscured the pineal but this could be displaced with the aspirator. Once visible the pineal was found to be of a distinctive pinkish grey colour.

The habenular commissure was divided on both sides of the pineal using a 23 gauge needle, after which, a pinealectomy or sham procedure was randomly determined.

3.4 Treatment for pinealectomy

Using a blunt 14 gauge needle the pineal was gently aspirated. Bleeding was found to vary from absent to considerable, depending on the number and size of vessels proximal to the pineal region that were damaged. If bleeding resulted haemostasis was achieved using Gelfoam and cotton tamponades, after which the resultant inter-hemispheric clot was gently removed by the aspirator. The fissure was filled with sterile normal saline allowing the deflected hemisphere to approximate it's normal position.

Having ensured haemostasis was achieved, the dural flap was replaced and sutured on the rostral, caudal and lateral dura margins using polyglycolic acid sutures (Dexon) and a 7mm reverse cutting needle (Fig. 3.4-1). The bone plate was replaced, but not sutured, and manipulated gently *in situ* to check for further bleeding (Fig. 3.4-2).

The skull was dried with cotton gauze and antibiotic powder (Tricin-V.R. Laboratories) applied to the rostral and caudal aspects of the wound. The skin was closed with wound clips (Totco Autoclips) and then sprayed with plastic surgical dressing (Nobecutane-B.D.H.). The procedure was usually completed within an hour, depending mainly on the time to achieve haemostasis.

3.5 Treatment for sham operation

Having divided both sides of the habenular commissure normal saline was applied to the fissure, the dura approximated and sutured, and the animal treated as described in the pinealectomy procedure.

All tammars subjected to surgery were given a 1ml injection of antibiotic (Aquacaine L/A Suspension-C.S.L.) and when recovered from anaesthesia the pouch young were returned to the pouch.

3.6 Operative care

(i) Pre-operative

Tammars were maintained for up to 20 days in warm natural daylight pens (see Chapter 2.1-(i)) prior to surgery and were visited daily to replenish food and water.

(ii) Post-operative

Immediately after surgery the animal was placed on a warming pad and observed for signs of respiratory distress. It was usually ambulant within two hours of completion of surgery and was returned to the holding pen. Further handling was avoided and animals were seen to take food and water by the day following surgery. Between two and seven days post-operative the animals could be moved to outside pens with other animals.

3.7 Adverse effects of surgery

Adverse effects of surgery were minimal. Extension of the neck was found in a few animals. This 'star gazing' attitude was probably due to damage to the superior colliculi(us) which are involved in visual location and lie caudal to the pineal. This, however, did not appear to affect the animals general vision, gait or appetite and such animals maintained excellent condition.

The incidence of deaths thought to be directly attributable to the surgical procedures is summarized in Table 3.7-1. Two deaths occurred within 30 minutes of induction of anaesthesia. It is possible hypersensitivity to the anaesthetic or un-noticed respiratory distress was at fault in these cases. Occasionally animals that had recovered from anaesthesia and were ambulant and taking food, were found dead in the postoperative pens. Examination of the brains of these animals revealed intracerebral haemorrhage in some cases. Spontaneous bleeding or bleeding as a result of trauma (possibly following collision with the cage when disturbed) may have accounted for these deaths. In the remaining animals intracerebral bleeding was not obvious and the cause of death was unknown.

3.8 Assessment of the procedures

To ensure the pineal gland was completely removed in pinealectomized animals, but undamaged and functional in sham-operated animals,

a series of adult female tammars were subjected to either the pinealectomy (N=6) or sham operation (N=6) procedures, sacrificed four months later, and the epithalamic region of their brain sectioned and examined for the presence of pineal tissue and damage to other neural structures. In addition blood samples were taken from these animals 20-28 days before and 29-37 days after surgery, every four hours for 24 hours, to monitor any changes in the circadian profile of plasma melatonin.

(i) Histological examination

Sections of the epithalamic region were examined by myself and Dr. L. Hinds independently, and summaries of these evaluations are shown in Table 3.8-1.

The treatment received by each animal, as evaluated by the examiners, is in accord with the intended surgical procedure. One animal (No. 560) was found to be incompletely pinealectomized. Damage to structures surrounding the epithalamic region was seen in eight animals. The degree of damage to other structures was considered sufficiently variable in both PINX and Sham PINX animals to exclude any reproductive effects that were not attributable to pinealectomy or sham operation. Examples of epithalamic sections of a sham pinealectomized and pinealectomized tammar are shown in Figures 3.8-2 and 3.8-3.

(ii) Melatonin profiles

The pre-and post-operative concentrations of plasma melatonin at each time point, for each animal, are given in Appendix A.1 and statistical analyses of the data are given in Appendix A.2.

A significant nocturnal rise in plasma melatonin was seen for all tammars preoperatively (Figure 3.8-4(A)). Pinealectomy abolished the nocturnal rise of melatonin in all animals, to undetectable levels (<31pg/ml) in four wallabies, and to daily mean levels of 44.71 $\pm$ 4.64 and 44.0 $\pm$ 3.55pg/ml (mean $\pm$ s.e.m.) for Numbers 520 and 528 (B, Appendix A.1). The dark values were not significantly different to light values in the PINX group ($P > 0.05$, Appendix A.2).

A significant nocturnal rise persisted in the sham operated group (C). For statistical analyses time points at which no melatonin was detectable are expressed as the level of sensitivity of that assay, i.e. either 16 or 31 picograms. The apparent elevated basal light concentrations in the pinealectomy profile (B), compared to the pre-operative (A) and sham (C) profiles, is due to these samples being assayed in a separate assay having a lower level of sensitivity.

3.9 Discussion

The described procedures for pinealectomy and sham-pinealectomy were found to be rapid and sufficiently reliable. Histological examination of the epithalamic region revealed inadvertent damage to tissue around the pineal gland which, although minimal, indicated that more care was needed during hemispheric displacement and aspiration of the pineal. No remnants of pineal tissue were found in pinealectomized animals other than Number 560 in which almost the whole pineal remained. The failure of this animal to exhibit a nocturnal rise of plasma melatonin may have been due to deafferentation of the gland by damage to the nerves providing sympathetic control of pineal function. This reinforced the need for extra care during the procedure, in isolating the organ from surrounding tissue before aspiration, and rechecking the site following supposed extirpation of the gland.

Abolition of the nocturnal rise of plasma melatonin following pinealectomy indicated that the major organ of melatonin synthesis in the tammar is the pineal gland; however, the presence of plasma melatonin at basal levels in two tammars postoperatively suggests that there may be an extra pineal source of melatonin.

The persistence of the nocturnal rise of melatonin in the sham-operated animals demonstrates that the sham procedure did not interfere with the circadian pattern of melatonin synthesis so, in terms of this indoleamine, the pineal remained functional. This finding was of paramount importance as it is reported that sham pinealectomy performed on rats under white light depressed the nocturnal pineal melatonin content by 50% when measured 25 days and 2 months postoperative, but rats operated on under red light differed only slightly from the intact control group (Frowein and Lapin, 1979).

Changes in the pre-and post-operative circadian profiles of melatonin proved to be an adequate determinant of the integrity of the pinealectomy or sham procedures and alone were considered sufficient to establish surgical integrity in subsequent experiments.

Figure 3.2-1: A tammar secured to the head support ready for surgery.

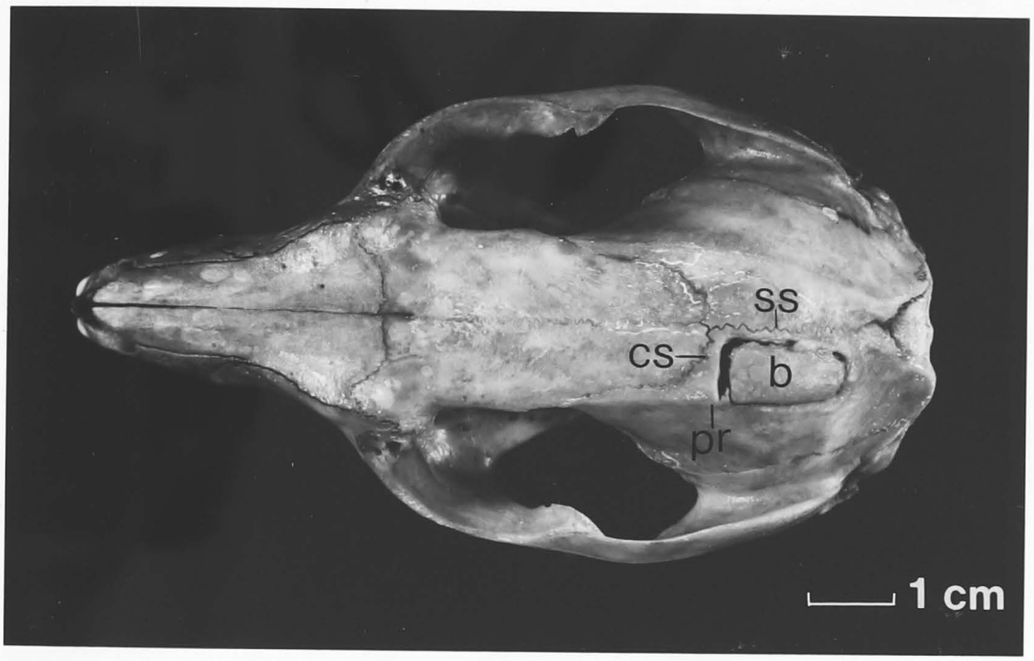


Figure 3.3-1: Dorsal view of a tammar skull showing the location of the bone plate which was removed to visualize the pineal gland. This animal (No. 5278) died 5-6 months after pinealectomy and the bone plate had fused with the skull.

cs - coronal suture
ss - sagittal suture
pr - parietal ridge
b - bone plate.

Figure 3.3-2: A formaldehyde fixed tammar brain cut in the mid-sagittal plane to show the position of the pineal gland relative to other neural structures.

hpc - hippocampal commissure

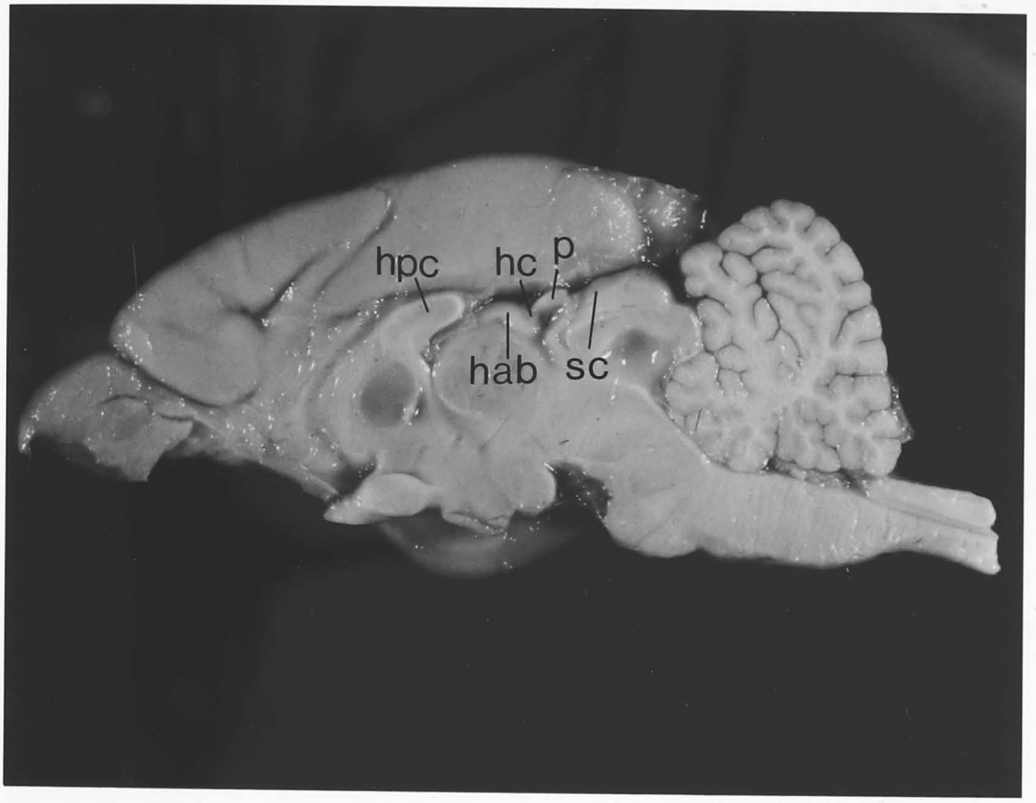
hc - habenular commissure

hab - habenular nucleus

sc - superior colliculus

p - pineal

Figure 3.3-3: Dorsal view of a tammar brain (formaldehyde fixed) with the left occipital cortex removed to expose neural structures in the epithalamic region. See above for abbreviations.


hpc
hc
p
hab
sc

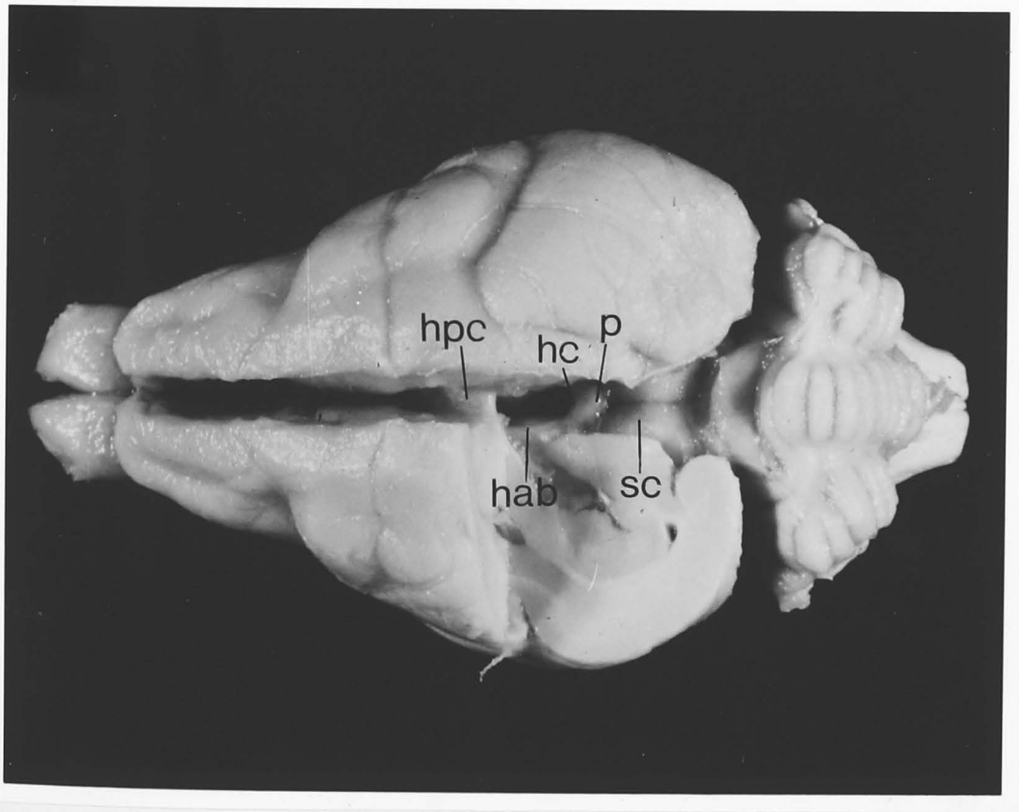

hpc
hc
p
hab
sc

Figure 3.4-1: The dural flap approximated and sutured to the dura margins.

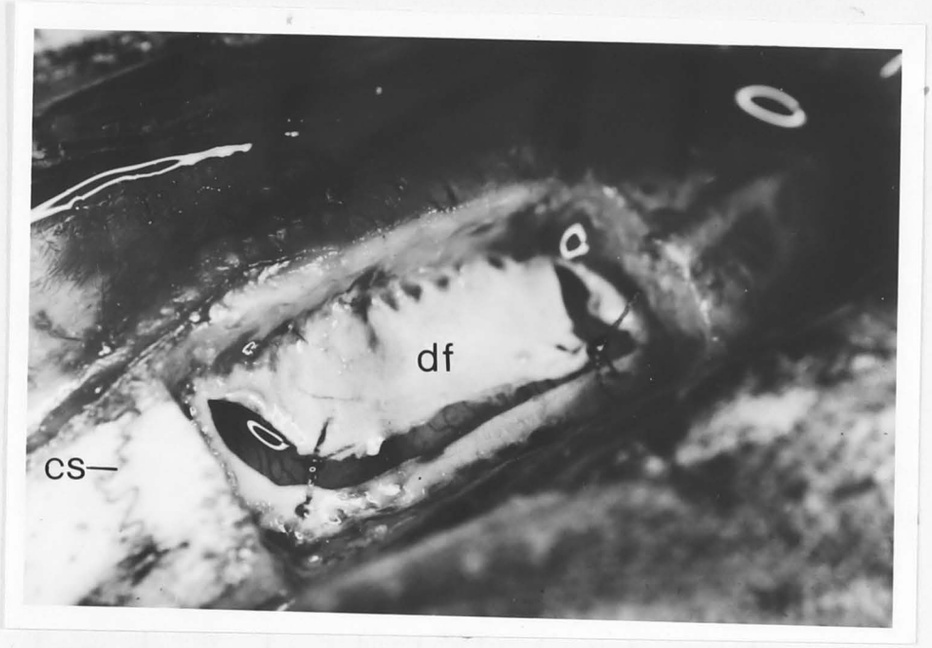
cs - coronal suture

df - dural flap

Figure 3.4-2: The bone plate replaced and supported by the underlying dura.

cs - coronal suture

b - bone plate


df
cs—

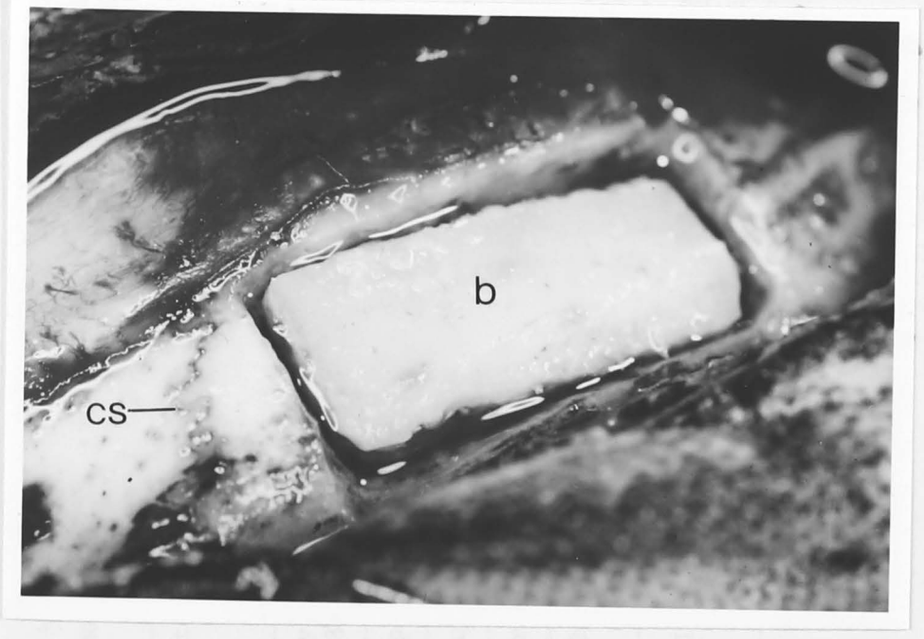

b
cs—

Table 3.7-1: The incidence and causes of mortality for tammars subjected to the pinealectomy or sham pinealectomy procedures throughout this study.

MORTALITY DETAILS							
					CAUSE OF DEATH		
SURGERY DATE	NUMBER OF OPERATIONS	ANIMAL NUMBER	PRIOR TREATMENT	TIME OF DEATH POST.OP.	Sacrificed (reason)	Intracerebral Haemorrhage	Unknown
October 1981	15	390	SHAM PINX	~24 hrs			X
		503	SHAM PINX	~48 hrs		X	
		505	SHAM PINX	56 hrs	X (emaciated)		
May/June 1982	12	5323	scalp incision	30 mins			X
September 1982	12	NIL					
December 1982	12	4889	SHAM PINX	~24 hrs			X
April 1983	10	5000	PINX	220 mins		X	
July 1983	13	4407	craniotomy	30 mins			X
		4920	SHAM PINX	<24 hrs		X	
TOTALS			PINX SHAM OTHER				
	74	8	1 5 2		1	3	4

Table 3.8-1: Evaluation of surgical procedures and trauma to the pineal region of pinealectomized and sham-operated tammars by two observers. Score: None (-); minimal (+); moderate (++); maximal (+++); pineal intact (0); pineal damaged (Ø)

ANIMAL NO.	OBSERVER NO.	PINEAL GLAND	PINEAL REMNANTS	DAMAGE TO				TREATMENT	
				EPITHALAMIC REGION		SUPERIOR COLLICULUS			
				LEFT	RIGHT	LEFT	RIGHT	EVALUATED	INTENDED
392	1	0	-	++	-	++	-	SHAM	SHAM
	2	0	-	++	-	++	-	SHAM	
502	1	-	-	++	-	-	-	PINX	PINX
	2	-	-	+	-	-	-	PINX	
504	1	-	-	+	-	-	-	PINX	PINX
	2	-	-	+	-	+	-	PINX	
506	1	-	-	-	-	-	-	PINX	PINX
	2	-	-	-	-	-	-	PINX	
510	1	0	-	+	-	-	-	SHAM	SHAM
	2	0	-	-	-	-	-	SHAM	
520	1	-	-	-	-	-	-	PINX	PINX
	2	-	-	-	-	-	-	PINX	
528	1	-	-	-	-	++	++	PINX	PINX
	2	-	-	-	-	++	++	PINX	
560	1	Ø	+++	-	-	-	-	failed PINX	PINX
	2	Ø	+++	++	-	++	-	failed PINX	
4700	1	0	-	-	-	-	-	SHAM	SHAM
	2	0	-	-	-	-	-	SHAM	
4772	1	0	-	+	-	-	-	SHAM	SHAM
	2	0	-	-	-	-	-	SHAM	
4950	1	Ø		-	-	-	-	failed SHAM	SHAM
	2	Ø	+++	+	-	-	-	failed PINX	
4954	1	0	-	+	-	-	-	SHAM	SHAM
	2	0	-	-	-	-	-	SHAM	

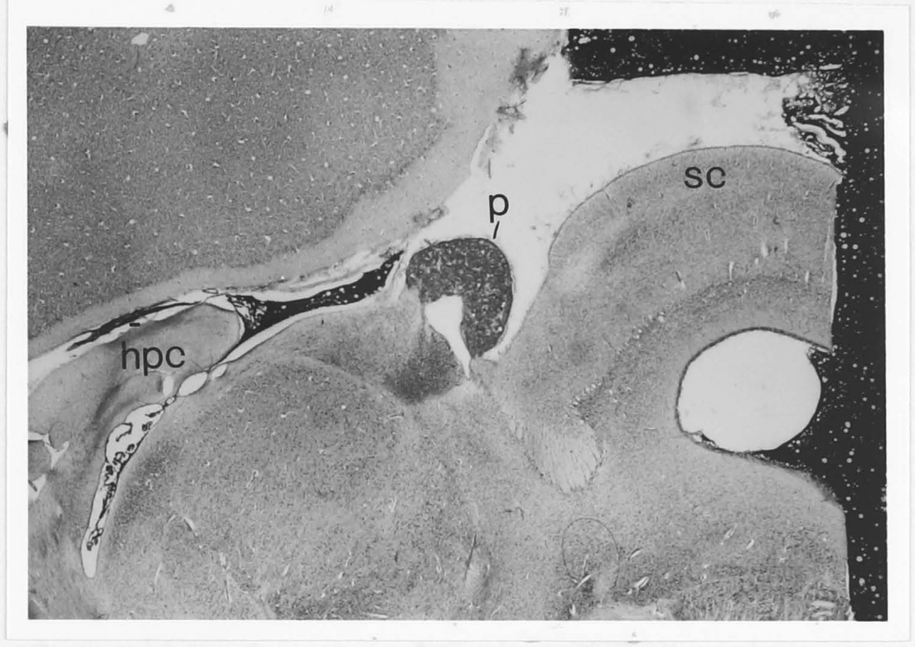
Figure 3.8-2: Parasagittal section of the epithalamic region of a sham pinealectomized tammar (No. 392).

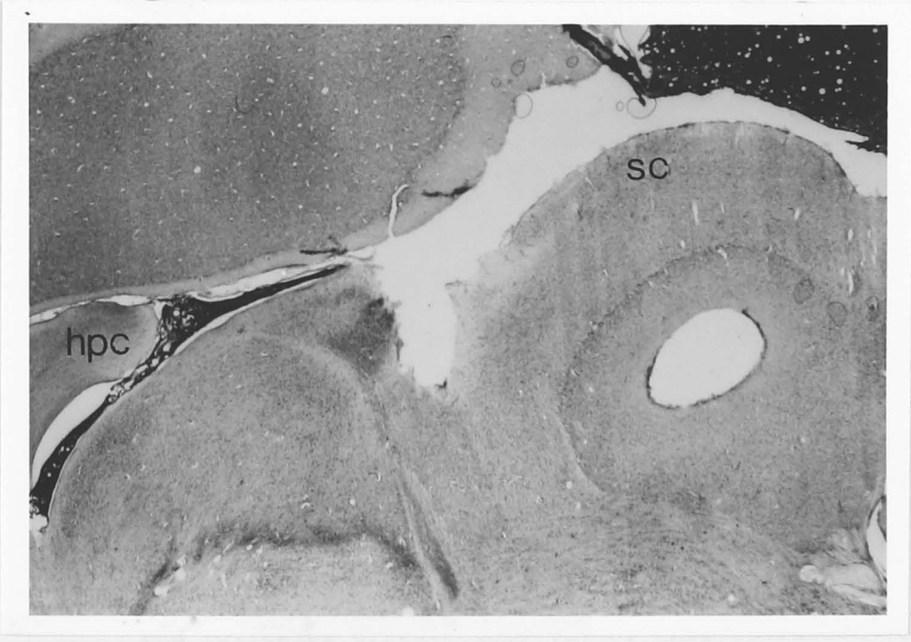
sc - superior colliculus

hpc - hippocampal commissure

p - pineal

Figure 3.8-3: Parasaggital section of the epithalamic region of a pinealectomized tammar (No. 520). See above legend for abbreviations.


sc
p
hpc


sc
hpc

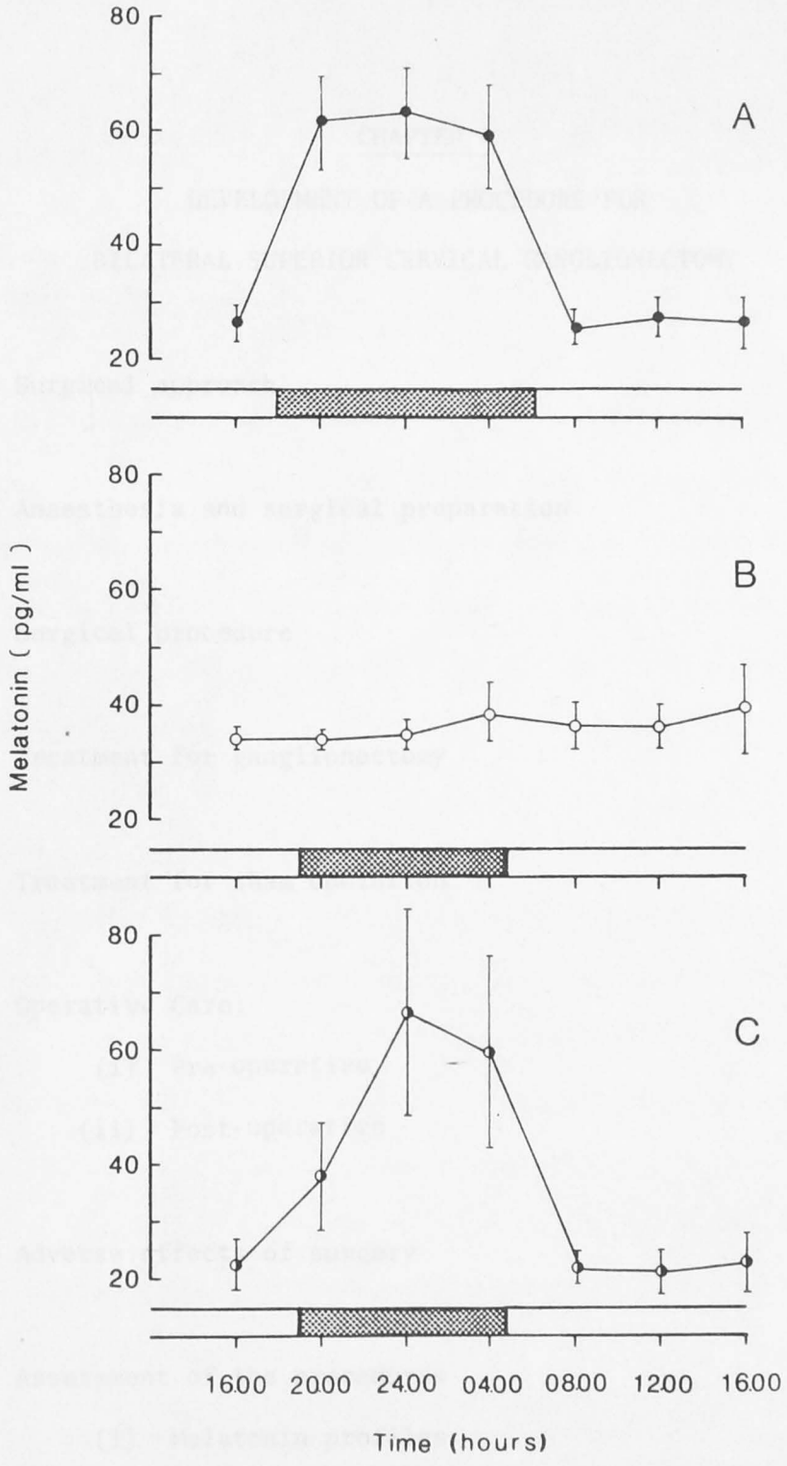


Figure 3.8-4: The circadian profile of plasma melatonin (pg/ml , mean ± s.e.m.) measured in tammars pre-operatively (A) and following pinealectomy (B) or sham operation (C). The stippled bars indicate the periods of lights off. Raw data and statistical analyses are given in Appendices A.1 and A.2.

CHAPTER 4

DEVELOPMENT OF A PROCEDURE FOR BILATERAL SUPERIOR CERVICAL GANGLIONECTOMY

4.1 Surgical approach

4.2 Anaesthesia and surgical preparation

4.3 Surgical procedure

4.4 Treatment for ganglionectomy

4.5 Treatment for sham operation

4.6 Operative Care:

(i) Pre-operative

(ii) Post-operative

4.7 Adverse effects of surgery

4.8 Assessment of the procedures

(i) Melatonin profiles

(ii) Tyrosine hydroxylase activity in excised tissue

(iii) Manifestation of ptosis.

4.9 Discussion

DEVELOPMENT OF A PROCEDURE FOR BILATERAL SUPERIOR CERVICAL GANGLIONECTOMY

4.1 Surgical approach

To locate and determine the least traumatic surgical approach to the superior cervical ganglion (SCG), a juvenile male tammar was sacrificed by intravenous overdose of anaesthetic and the structures of the neck and upper thorax exposed. The musculature and blood vessels of the neck were identified and a 70% alcohol solution was applied to the region to make the nerves opaque.

The carotid sheath was examined carefully to identify the common carotid artery, internal jugular vein, the vagus nerve and sympathetic trunk approximating the vagus nerve. The vagus nerve was found to be thicker, with a striated appearance, whilst the sympathetic trunk was less discrete and gave the appearance of being composed of several fibres. Both the vagus nerve and sympathetic trunk were followed rostrally to either the vagus ganglion or the SCG. The SCG was found deeper and more medial than the vagus ganglion and slightly rostral to the angle of the mandible.

Having established the location of the SCG, trial sympathectomies were performed on two adult female tammars which proved to be successful. (See results, Section 4.8, for animals No. 637 and 694).

4.2 Anaesthesia and surgical preparation

Following fasting overnight the animal was weighed and anaesthesia induced and maintained by infusion of a 4% solution of

sodium thiamylal (Surital) into the lateral tail vein, followed by 0.12mg in 0.2ml of Atropine Sulphate. The neck region was shaved and scrubbed with a 1% solution of Cetavlon. Lying supine on a warming pad ($37^{o}C$), the head was extended using thin cord secured behind the upper incisors.

4.3 Surgical procedure

A midline incision was made in the skin extending from a point 1cm rostral of the mandibular angle to 2cm caudal of the larynx, and the wound held open using retractors. The parotid gland was freed from adherent connective tissue and retracted laterally using towel forceps. By blunt dissection between the sternohyoideus/omohyoideus and sternocleidomastoideus muscles, the mandibular angle was exposed rostrally, and the carotid sheath caudally. The carotid sheath was examined, the sympathetic trunk and vagus nerve identified, and the SCG located. Having located the rostral and caudal aspects of the ganglion, a ganglionectomy or sham procedure was determined randomly and the procedure repeated for the contralateral ganglion.

4.4 Treatment for ganglionectomy

The ganglion was grasped with forceps and dissected out by cutting the afferent and efferent fibres with fine iridectomy scissors. The wound was dusted with antibiotic powder (Tricin), approximated with wound clips, and sprayed with a plastic wound dressing (Nobecutane). One ml of a long acting antibiotic (Aquacaine L/A Suspension) was given intramuscularly. The procedure could be completed within 45-60 minutes.

4.5 Treatment for sham operation

Having located the rostral and caudal aspects of the superior cervical ganglion, it was palpated and the wound closed as previously described.

4.6 Operative care

(i) Pre-operative

Four days before the series of operations began the tammars were transferred from outside yards to the natural daylight pens described in Chapter 2.1-(i).

(ii) Post-operative

The animal was placed on a warming pad following surgery and respiration monitored closely for signs of distress. Following recovery from anaesthesia, the animal was returned to the pens already described, and its general condition checked daily until transferred to outside yards.

4.7 Adverse effects of surgery

Following excision of both ganglia some animals developed respiratory complications (Table 4.7-1) and these required constant attention until recovery from anaesthesia. Aspiration of saliva and

extension of the neck and tongue were often necessary to ensure ease of ventilation. Following recovery from anaesthesia, however, these animals were found to breatheand behave normally.

Three of 15 tammars were sacrificed due to complications as a result of surgery (Table 4.7-2). These were accidental severing of the carotid artery resulting in uncontrollable haemorrhage, respiratory incompetence resulting in cyanosis and possibly brain damage, and an excessive decline in weight. These complications were not confined to a particular treatment.

4.8 Assessment of the procedures

To confirm the SCG had been completely removed and the pineal denervated in ganglionectomized tammars, but that the pineal retained sympathetic innervation in sham operated animals, the following observations were made:

(i) Melatonin profiles

Blood samples were taken during mid-light and mid-dark on the same day 4-7 days before and 130-140 days after surgery. Plasma melatonin levels were measured by radioimmunoassay. Melatonin levels measured pre-operatively were significantly higher during the dark compared to those during the light. This nocturnal rise persisted in each Sham SCGX animal but was abolished in all of the SCGX animals (Figure 4.8-1 and Table 4.8-2).

(ii) Tyrosine hydroxylase activity in excised tissue

To ensure the excised tissue contained adrenergic ganglion cells, tyrosine hydroxylase (T-OH) activity was measured by radiochemical enzyme assay (Hendry and Iversen, 1971) by Dr. I. McLennan (Department of Behavioural Biology, R.S.B.S., Australian National University). T-OH is localised in adrenergic ganglion cells (Black *et al.*, 1971) and is rate-limiting in catecholamine biosynthesis (Levitt *et al.*, 1965). T-OH activity was recorded in all tissue samples assayed (Table 4.8-3).

(iii) Manifestation of ptosis

Post-ganglionic fibres of the SCG supply vasoconstrictor and sudomotor nerves to the head. Bilateral sympathectomy results in Horner's syndrome, characterized by drooping of the upper eyelid (ptosis) amongst other less obvious symptoms (Williams and Warwick, 1980). Following complete recovery from anaesthesia each animal was examined for the manifestation of ptosis in each eye (Table 4.8-4). Ptosis was obvious in each eye of all animals (with the exception of No. 5085), but was less obvious after the following day. No ptosis was noticed in the Sham SCGX animals.

4.9 Discussion

The method for superior cervical ganglionectomy described here was found to be a relatively simple procedure compared to the standard procedure described in the sheep (Appleton and Waites, 1957), which

requires bilateral incisions through the cervical skin and musculature. Complications associated with the procedures were respiratory distress seen in three animals, and the excessive weight loss of one animal. The respiratory distress may have been due to denervation of some respiratory structure by excision of the SCG, or inadvertent damage to nerves of vagal origin. In primates, medial branches of the SCG are the larynogpharyngeal branches that, together with branches from the glosso-pharyngeal and vagus nerves, form the pharyngeal plexus. This provides both motor and sensory elements to the pharynx (Williams and Warwick, 1980). Respiratory difficulty after bilateral SCGX has also been reported in the mare (Sharp, Vernon and Zavy, 1979).

The excessive weight loss seen in one animal may have been due to incompetent digestion resulting from damage to the vagus nerve, as branches of the vagus nerve provide secretory and motor influences on the stomach and intestine, and vagotomy causes the stomach to become flaccid and to empty slowly (Williams and Warwick, 1980).

The tyrosine hydroxylase levels measured in excised tissue confirmed removal of adrenergic ganglia and the manifestation of ptosis confirmed lack of post-ganglionic innervation to the upper eyelid. Although ptosis was observed immediately post-operatively, it was not as obvious 24 hours later. These animals were therefore able to compensate for this symptom, which also may explain the absence of ptosis in one SCGX animal. The lack of ptosis in sham operated animals indicates the post-ganglionic fibres were left intact. Slight ptosis was reported in SCGX Soay rams (Lincoln, 1979), but was not definite in SCGX goats (Buttle, 1977) and was not used for confirmation of surgery in the goats.

Bilateral SCGX abolished the nocturnal rise of plasma melatonin, confirming denervation of the pineal gland, as previously reported for the tammar (Renfree *et al.*, 1981) and the Soay ram (Lincoln, Almeida, Klandorf and Cunningham, 1982). SCGX of another marsupial, the potoroo, also caused anatomical changes in the pineal (Bradley, 1973). The persistence of the nocturnal elevation in sham operated tammars shows that, in terms of melatonin synthesis, pineal gland function was unaffected by this procedure.

Table 4.7-1: Complications associated with bilateral superior cervical ganglionectomy (SCGX)

Month, Year	Number of Operations	Animal Number	Treatment	Complications	
				During Surgery	Post-operative
October, 1982	15	637	bilateral SCGX	Laboured breathing	none
October, 1982		5085	bilateral SCGX	Hoarse breathing	none

Table 4.7-2: The incidence of mortality in tammars subjected to SCGX or Sham SCGX procedures

Month, Year	Number of Operations	Animal Number	Prior Treatment	Time of death post-op.	Symptoms/ Condition	Cause of Death
October, 1982	15	623	incision	50mins.	carotid artery severed	sacrificed
		691	bilateral SCGX	20hours	cyanosis, unable to stand	sacrificed
		672	Sham SCGX	6 days	weight loss, excessive salivation	sacrificed

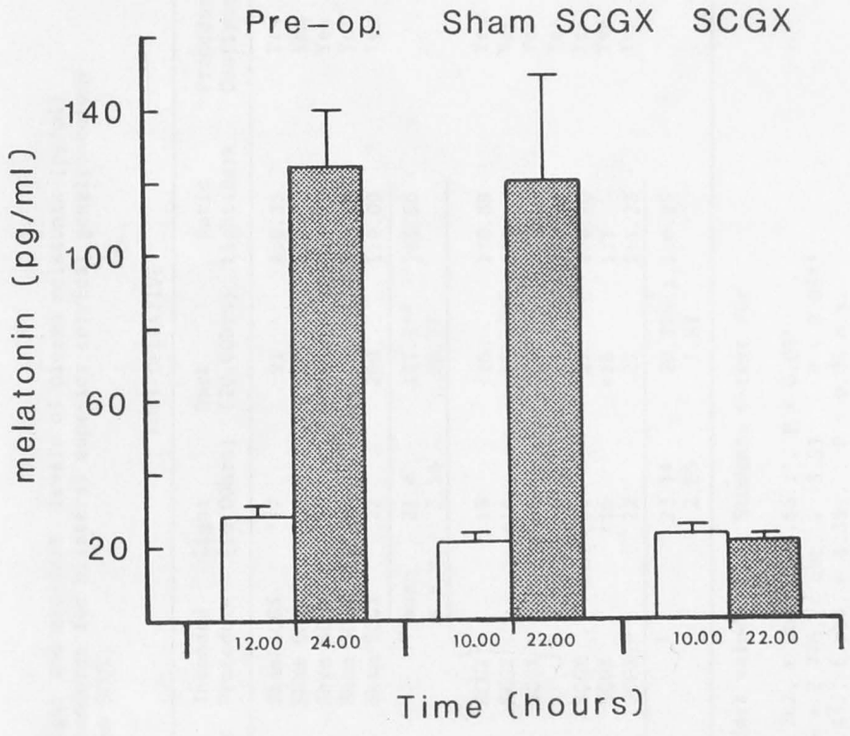


Figure 4.8-1: Pre- and post-operative mid-light and mid-dark concentrations of plasma melatonin in SCGX and Sham SCGX tammars. Values given are the mean ± s.e.m. Raw data and statistical comparisons are given in Table 4.8-2. Stippled bars indicate samples taken during the dark phase.

Table 4.8-2: Pre-and post-operative mid-light and mid-dark levels of plasma melatonin (pg/ml) in tammars subjected to procedures for bilateral superior cervical ganglionectomy (SCGX) or sham operation (Sham SCGX)

	PRE-OPERATIVE				POST-OPERATIVE			
Animal Number	Light (12.00hrs)	Dark (24.00hrs)	Ratio Light:Dark	Intended Procedure	Light (10.00hrs)	Dark (20.00hrs)	Ratio Light:Dark	Procedure Confirmed
526	34	110	1:3.24	Sham SCGX	17	91	1:5.35	Yes
618	46	178	1:3.87	Sham SCGX	25	122	1:4.88	Yes
641	17	170	1:10	Sham SCGX	27	165	1:6.11	Yes
693	16	47	1:2.94	Sham SCGX	16	28	1:1.75	Yes
5597	29	94	1:3.24	Sham SCGX	22	200	1:9.09	Yes
				mean	21.4	121.2**	1:5.66	
				s.e.m.	2.16	29.77		
617	33	92	1:2.79	SCGX	18	<16	1:0.89	Yes
637	no samples			SCGX	<16	<16	1:1	Yes
654	30	200	1:6.67	SCGX	31	19	1:0.61	Yes
694	no samples				29	21	1:0.72	Yes
4670	34	175	1:5.15	SCGX	30	27	1:0.90	Yes
5085	16	100	1:6.25	SCGX	<16	<16	1:1	Yes
654	29	76	1:2.62	SCGX	22	27	1:1.23	Yes
mean	28.4	124.2***	1:4.37		23.14	20.29n.s.	1:0.88	
s.e.m.	3.04	16.44			2.55	1.87		

Statistical comparisons between light and dark values using Students t-test for dependent samples.
Both groups pre-operative: t 0.05, 9 = $\pm$ 2.262, t obt. = -5.55 $\therefore$ $P < 0.05$*
Sham SCGX group post-operative: t 0.05, 4 = $\pm$ 2.776, t obt. = -3.53 $\therefore$ $P < 0.05$**
SCGX group post-operative: t 0.05, 6 = $\pm$ 2.447, t obt. = 1.35 $\therefore$ $P > 0.05$ n.s.

Table 4.8-3: Tyrosine hydroxylase (T-OH) levels measured in excised tissue of bilateral superior cervical ganglionectomized tammars. Blank values (60nmol x hr^{-1}) have been deducted from the values given.

Animal Number	T-OH (nmol x hr^{-1} x $sample^{-1}$) Left Side	Right Side
617	618	559
637	207	70
654	793	583
658	260	1128
694	samples destroyed	
4670	870	1158
5085	548	653

Table 4.8-4: Determination of the manifestation of ptosis in SCGX and Sham SCGX tammars

Animal Number	Left Eye	Right Eye	Treatment
526	-	-	Sham SCGX
618	-	-	Sham SCGX
641	-	-	Sham SCGX
693	-	-	Sham SCGX
5597	-	-	Sham SCGX
617	+	+	SCGX
637	+	+	SCGX
654	+	+	SCGX
658	+	+	SCGX
694	+	+	SCGX
4670	+	+	SCGX
5085	*	*	SCGX

\+ ptosis present

\- ptosis absent

* not obvious.

CHAPTER FIVE

RADIOIMMUNOASSAY FOR MELATONIN

5.1 Principles of the radioimmunoassay

5.2 Reagents

(i) Buffer solutions

(ii) Standards

(iii) Radioligand

(iv) Antiserum

(v) Separation solutions

(vi) Scintillation fluid

5.3 Development of the radioimmunoassay

(i) Antiserum titration

(ii) Assay precision

(iii) Extraction efficiency

(iv) Sensitivity

(v) Assay procedure

5.4 Assay validation for the tammar

(i) Parallelism of dose response curves

(ii) Recovery of exogenous melatonin

(iii) Intra- and interassay variation

(iv) Melatonin levels in pinealectomized tammars

RADIOIMMUNOASSAY FOR MELATONIN

5.1 Principles of the radioimmunoassay

Radioimmunoassay (RIA) techniques have allowed measurement of hormones that occur in very low concentrations in body fluids. The RIA is one of several types of competitive binding assays that utilizes four basic reagents: ligand, radioligand, binder and separator (Ransom, 1976). The ligand is the substance or hormone that is to be measured in the assay. It has two functions. Alone, or conjugated with other immunostimulating molecules, it is used to raise antibodies that have a specific affinity to the ligand and, secondly, it competes in the assay with radioligand for binding sites on these antibodies. The radioligand is ligand that has a radioactive element incorporated into its structure. The binder is an antibody that is raised against the ligand and has a specific affinity for both the ligand and radioligand. The separator is a substance which also has an affinity for both ligand and radioligand that are not bound to the binder.

The RIA is based on the competition between the ligand and radioligand for a limited number of binding sites on the antibodies (antiserum). As the concentration of ligand increases, radioligand is displaced from the antibody sites. Following equilibration of this reaction, unbound ligand and radioligand are removed from the assay medium by the separator, leaving only ligand and radioligand that are bound to the antibodies. The ratio of the remaining bound ligand to radioligand is dependent on the amount of each present during incubation. As the amount of radioligand is constant within the assay, differing concentrations of ligand added will result in differing amounts

of radioligand being bound by the antibody. As the radioactivity of the radioligand can be quantified, a measure of the amount of radioligand bound following separation can be determined.

A series of known concentrations of ligand (standards) are included in the assay. Low concentrations of ligand leave more binding sites for the radioligand, and so more radioactivity is bound in these standards following separation. Standards having higher ligand concentrations leave less binding sites for the radioligand and so less radioactivity is detected. The amount of ligand (or hormone) in unknown samples can therefore be determined by reference to the standards.

The routine use of a previously described assay in a new laboratory, and for a new species, requires that the final assay characteristics conform to those described for the original assay, and that the assay is validated for use in the new species. The following procedures were performed, and parameters monitored, to achieve these aims:

(i) determine the efficiency of extraction of the hormone from plasma and buffer,

(ii) demonstrate quantitatively correct recovery of the hormone,

(iii) demonstrate that non-specific factors in plasma of the new species do not interfere in the assay,

(iv) determine assay sensitivity (the smallest amount of hormone distinguishable from no hormone),

(v) determine and monitor assay precision (the variation in estimation of a hormone within and between assays),

(vi) determine and monitor blank values (the amount of hormone determined for samples containing no hormone) and

(vii) demonstrate that measured changes in the hormone concentration conform with the animals' physiological status, e.g. removal of the organ producing the hormone depresses levels of the hormone.

The protocol for the melatonin RIA presented here was based on that of Kennaway *et al.* (1982) and Kennaway (*pers. comm.*), with minor modifications to suit the facilities provided at Dr. Tyndale-Biscoe's laboratory at the CSIRO Division of Wildlife and Rangelands Research, Canberra. All solutions used in assay were those according to Dr. Kennaway's protocol, except where specified otherwise.

5.2 Reagents

All chemicals used were laboratory grade from Ajax, B.D.H., May and Baker, New England Nuclear, Sigma or Pharmacia Fine Chemicals.

(i) Buffer solutions:

Assay buffer. The assay buffer was prepared from:

- 14.2gm $Na_2HPO_4.2H_2O$,
- 2.9gm $NaH_2PO_4.2H_2O$,
- 8.7gm NaCl,
- 1.0gm NaN_3,
- 5.0gm Bovine Serum Albumin (Fraction V),
- 0.5gm Bovine Gamma Globulins (Cohn Fraction II),
- one litre distilled water,
- pH was corrected to 7.4.

Borate buffer. The borate buffer was prepared from:

- 152gm $K_2B_4O_7.4H_2O$,
- one litre distilled water,
- pH was corrected to 9.6.

(ii) Standards:

The melatonin was purchased from Sigma Chemicals and standard curves in the range of 0 to 1000pg were constructed in either of two ways. The reason for this, and the procedures, are discussed in Section 5.3(ii).

(iii) Radioligand:

The [^{3}H] melatonin radioligand (tracer) was purchased from New England Nuclear and had a specific activity of 30.4Ci/mmol. Twenty microlitres was transferred to one ml absolute ethanol (1:50). The 1:50 stock solution was stored at 4^{o}C and discarded after 14 days.

A working dilution of tracer was prepared by diluting the stock 1:100 or 1:150 with assay buffer. 100µl was added to each tube.

(iv) Antiserum:

The antiserum to melatonin (G280) was kindly provided by Dr. Kennaway. It was raised in a goat against a melatonin-bovine γ-globulin conjugate. Cross-reactivities at the 50% displacement level were determined by the supplier (Kennaway *et al.* 1982) to be:

- 6-hydroxymelatonin, 0.02%
- 6-sulfatoxymelatonin, 0.3%
- N-acetyl-serotonin, 0.3%
- N-acetyl-tryptamine, 0.4%
- 5-methoxytryptamine, 0.01%
- 5-methoxytryptophol, 0.01%
- serotonin, 0.10%
- 0-acetyl-5-methoxy-trypthophol, 0.06%.

Stock antiserum: fifty milligrams of the lyophylized antiserum was dissolved in 12.5ml assay buffer to give an initial dilution of 1:25 (assuming 100mg solid per ml in serum).

Fifty microlitre aliquots of the 1:25 solution were frozen at -15^{o}C to be used in all subsequent assays.

Working dilutions:

The 1:25 stock aliquots were further diluted for use in the assay. As several dilutions were used in establishing the assay these will be discussed in the relevant subsequent sections.

(v) Separation solutions

Saturated ammonium sulphate. This was prepared from:

- 520gm ammonium sulphate
- made up to one litre with distilled water.

Charcoal-dextran coated. This was prepared from:

- 6.25gm Norit A - activated charcoal
- 0.625gm Dextran T-70
- 250ml distilled water.

The charcoal was washed with distilled water and after settling the fines were aspirated off. This was repeated five times and finally made up to 100ml with distilled water.

For use in the assay this stock solution was diluted 1:10 with assay buffer.

(vi) Scintillation Fluid:

The scintillation fluid was prepared from:

- 42ml Liquifluor,
- 150ml 1-4 Dioxan,
- 1 litre toluene.

5.3 Development of the radioimmunoassay

The following section describes procedures that were performed to establish the assay at CSIRO. In general the protocol of Dr. Kennaway was followed but minor modifications were made when considered necessary. These will be described in detail.

(i) Antiserum titration:

The sensitivity of an assay is dependent on a number of factors (Midgley *et al.* 1969), one of which is the affinity of the antiserum for the ligand and radioligand. In practice the concentration of antiserum that binds about 50% of the radioligand is the optimal concentration to obtain the highest sensitivity (Ransom, 1976). To determine the amount of antiserum G280 that would bind 50% of the tracer several dilutions of G280 were tested. This titration was set up as follows:

Standards

Three series of assay tubes in duplicate containing 0, 10, 50, 100 or 500pg of melatonin were prepared according to Dr Kennaway's original protocol in 500µl assay buffer (Table 5.3-4).

Antiserum

For each series a different working dilution of antiserum was prepared in assay buffer (Table 5.3-1) and 100µl of the working dilution was added to each tube.

Tracer

To all tubes 100µl of [^{3}H] melatonin (1:100 working dilution) was added which gave approximately 15,000 counts per minute (cpm). All tubes received a further 100µl of assay buffer to wash down the reagents. Following incubation at 4^{o}C for 14 hours the antibody-melatonin fraction was precipitated by incubation with 200µl cold ammonium sulphate solution (4^{o}C) for 30 minutes, and centrifugation at 2,000rpm for 15 minutes. The supernatant was aspirated and the remaining pellet dissolved in 1ml distilled water by vortex. A 750µl aliquot of the resuspended precipitate was added to 10ml scintillation fluid and the radioactivity counted in a scintillation counter after 24 hours quenching.

Non-specific binding (NSB)

For each series, two tubes containing

- 500µl assay buffer,
- 200µl assay buffer without antiserum
- 100µl [^{3}H] melatonin (1:100)

were included and treated as described for the standards to determine NSB (binding of tracer to non-specific factors of the medium, in the absence of antiserum).

Totals

To determine the total amount of tracer added to each tube during incubation a further series of tubes containing

. 900µl assay buffer and

. 100µl [^{3}H] melatonin (1:100)

were included but not separated. A 750µl aliquot was transferred to scintillation fluid and counted after 24 hours quenching.

For each standard the radioactivity bound (B) minus NSB was expressed as a percentage of the radioactivity of the totals (T), i.e. B-NSB/Tx100. The resultant displacement curves are shown in Figure 5.3-2.

The amount of radioligand bound in the absence of ligand (zero standard - Bo) was highest in the 1:3000 working dilution, and Bo decreased with increasing dilution of antiserum. The desired Bo of around 50% was seen in the 1:5000 working dilution. This is a slightly less dilution than that used in the original protocol to obtain the same binding (1:7500). In the presence of melatonin standard, less binding was found and this decreased with increasing amounts of melatonin standard, indicating that competition for binding sites between ligand and radioligand had occurred.

(ii) Assay precision:

The difference between duplicates was relatively high for some standards (Table 5.3-3) which was considered unsuitable for routine application of the assay. As this was probably due to methodological errors, original assay procedures that included many steps and therefore greater chances of error, were replaced by, and compared to, simpler procedures. Two procedures that were considered to be likely causes of

high variation were the methods for setting up the standard curve and separating the bound and free ligand and radioligand.

The standard curve utilized in the original protocol was made up from 2 stock standards. Each standard was aliquoted by a Hamilton syringe and made up to volume (500µl) with assay buffer for each tube. Because of the possibility of aliquoting error with the Hamilton syringe, a standard curve across the same range was made up by double dilution of one stock standard with no corrections for volume. A comparison of the procedures used to make up the original and new standard curves is given in Table 5.3-4. The replicates of each standard could then be taken from the same standard pool using the double dilution procedure, and the whole range of standards were relative, as each was determined by the previous standard. This procedure may have been more reliable in producing replicates of the same standard than in the original protocol where each replicate was made up separately.

In the original protocol, the pellet produced by precipitation of the Bovine Gamma Globulins/G280 complex and centrifugation was found, even with great care, to be capable of partial dislodgement during aspiration. As this pellet contained bound radioligand, loss of part of the pellet would have given an inaccurate determination of bound radioligand for that tube. An alternative procedure for separation of the bound and unbound fractions utilizes absorption of the unbound ligand and radioligand by charcoal particles. To make the absorptive capacity of the charcoal more selective it is coated with dextran (Lau, Gottlieb and Herbert, 1966), although the importance of this in separation has

been questioned by some workers (Yalow and Berson, 1971). A charcoal separation technique was used, together with the new standard curve, to compare variation in replicates with the original ammonium sulphate separation procedure.

Comparison of charcoal and ammonium sulphate separation techniques

Standards. Two standard curves in duplicate were set up in assay buffer (500µl per tube) according to the new double dilution procedure. To each tube was added

- 100µl antiserum (1:5000 working dilution),
- 100µl [^{3}H] melatonin (1:100 working dilution),
- 100µl assay buffer washdown.

Following incubation for 14 hours at 4^{o}C, the bound and unbound fractions were separated by addition of either

(i) 200µl saturated ammonium sulphate (4^{o}C) for 30 minutes, or

(ii) 200µl 0.625% dextran coated charcoal (4^{o}C) for 30 minutes, and centrifuged at 2,000 rpm for 15 minutes (ammonium sulphate series) or 6 minutes (charcoal series).

The supernatant of the ammonium sulphate series was aspirated off and 1ml distilled water added to resuspend the pellet. A 750µl aliquot was transferred to 10ml scintillation fluid and counted after 24 hours quenching.

Following centrifugation 750µl supernatant of the charcoal series was also counted as described. The displacement curves following ammonium sulphate and charcoal separation are shown in Figure 5.3-5 and the B/T for each standard is given in Table 5.3-6. The duplicates of each standard obtained following charcoal separation were less variable, as can be seen from the calculated standard deviations, than those obtained following ammonium sulphate separation. As the standards for each curve were both made up using double dilution, the higher variations for each standard following precipitation with ammonium sulphate could be attributed to this separation technique. The charcoal separation procedure was therefore considered a better technique and was used subsequently in the assay.

(iii) Extraction efficiency

The efficiency of extraction of melatonin from plasma and buffer was determined as follows:

. 900µl charcoal stripped tammar plasma or assay buffer was aliquoted into two series of 15ml extraction tubes (Quickfit).

. 100 µl [^{3}H] melatonin was added to each

. 1 ml borate buffer was added to each tube which, due to the high pH, reduced extraction of N-acetylserotonin and so cross-reactivity with this precursor to melatonin (Kennaway, *pers.comm.*).

As in the original protocol, lipid was extracted by the addition of 6ml petroleum spirit (B.R. 60-80^{o}C) and rocking at 70 excursions per minute for 30 minutes on a Dynamax flask shaker. Following centrifugation (2000 rpm/6 mins) the lipid interface and solvent were

aspirated off. The melatonin was then extracted with 6ml dichloromethane hexane (1:1) by rocking at 70 excursions per minute for 30 minutes. Following centrifugation, inverting the tubes twice and recentrifuging, 5 mls of the solvent was transferred to glass assay tubes and evaporated under industrial grade nitrogen in a 37°C water bath. Following evaporation, 300µl of methanol was added to each tube, vortexed, and evaporated under nitrogen at 37°C.

One ml of 1% ethanol assay buffer (ethanol was included to dissolve the crystalline melatonin) was added to each tube and following vortex and one hour equilibration, 750µl was aliquoted to scintillation vials and counted after 24 hours quenching. A third series of tubes containing 100µl [^{3}H] melatonin and 900µl 1% ethanol buffer (Totals) were not extracted, and 750µl was aliquoted from each and also counted. The extraction efficiency was determined as the number of counts in each tube expressed as a percentage of that determined in the totals. (Table 5.3-7).

The efficiency of extraction of [^{3}H] melatonin was less than the 80% reported by Kennaway *et al.* (1982), and there was less recovery from plasma. Several factors could have contributed to this low extraction efficiency:

1) emulsion formation was observed in some tubes during the lipid extraction. Although this may have accounted for low extraction efficiency in these, it did not explain the low level of all tubes.

2) a different flask shaker was used to that in the original protocol. Although the number of excursions was the same,the vigour of the shaking may not have been identical.

To improve recovery the following changes to the extraction procedure were made:

- a decrease in the number of rocks per minute during the lipid extraction to prevent emulsion formation and therefore possible variation in efficiency resulting from this.
- an increase in the number of rocks per minute during melatonin extraction.
- removal of all the dichloromethane: hexane solvent by first freezing the aqueous layer in a solid CO_2/ethanol bath and then pouring off the solvent. This meant all of the solvent was removed not 5 of the 6 mls as in the original protocol.
- eliminate the methanol washdown. If the above changes increased recovery to expected levels, the time and expense involved in the methanol washdown would not be necessary.

To determine the efficiency of extraction of melatonin from buffer and plasma over a range of melatonin concentrations using this new protocol, standards were made up in absolute ethanol (0, 31, 125, 500pg), and aliquoted in triplicate into two series of extraction tubes. 100µl [^{3}H] melatonin (15,000cpm in ethanol were added to each and the ethanol evaporated. One ml assay buffer was added to each tube of the first series and one ml charcoal stripped tammar plasma to each of the

second series. After addition of 1ml borate buffer, vortexing, and equilibration for one hour at room temperature, both series were extracted as follows:

Lipid extraction - 6ml petroleum spirit (B.R. 60-80^{o}C); 48 rocks per minute for 30 minutes. After centrifugation (2,000 rpm for 6 minutes) the lipid layer interface and solvent were aspirated off.

Melatonin extraction - 6ml dichloromethane:hexane (1:1); 140 rocks per minute for 30 minutes. After separation of the aqueous and solvent phases by standing for 15 minutes, the aqueous phase was frozen in a solid CO_2/ethanol bath, and the solvent decanted into glass assay tubes and evaporated.

One ml of 1% ethanol assay buffer was added to all tubes and, after vortex and equilibration for 1 hour at room temperature, 750µl was transferred to scintillation vials and counted after 24 hours quenching. A third series of tubes (Totals) containing no melatonin also received 100µl [^{3}H] melatonin in ethanol, which was evaporated and resuspended in 1ml 1% ethanol buffer. A 750µl aliquot was also counted.

The radioactivity determined for each of the buffer and plasma series was expressed as a percentage of the radioactivity added (Totals), and was found to be similar across the range of melatonin concentrations tested, and for both a buffer and plasma medium (Table 5.3-8). These recoveries of labelled melatonin were consistent with those reported by Kennaway *et al.* (1982), and this new extraction procedure was therefore used for subsequent assays.

(iv) Sensitivity

Assay sensitivity is defined as being the smallest amount of unlabelled hormone that is distinguishable from no hormone. In practice, assay sensitivity is calculated as being two standard deviations of the lowest standard failing to overlap with two standard deviations of the zero standard. Optimal sensitivity is obtained where the ratio of antiserum to tracer is such that a minimum number of binding sites are available for the least amount of tracer added, yet sufficient tracer is included to retain counting accuracy.

From the antiserum titration a 1:5000 working dilution of antiserum with a 1:100 working dilution of [^{3}H] melatonin gave a mean maximum binding of 56.04 $\pm$ 4.7% (Figure 5.3-2). To compare ratios of antiserum to tracer that would provide optimal sensitivity following extraction, the amount of antiserum was (i) increased, retaining the 1:100 dilution of [^{3}H] melatonin, and (ii) decreased with a corresponding decrease in [^{3}H] melatonin to 1:150 working dilution as shown in Table 5.3-9. In addition, the original protocol required dissolving of the crystalline melatonin extracted from samples in 1% ethanol assay buffer so, as the addition of ethanol may have interfered with binding, it was necessary to monitor any changes in binding by the inclusion of ethanol.

Standards

Two series of standard curves in buffer (0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000pg/ml) were set up in triplicate and 1 ml borate buffer was added to each tube. Following extraction

according to the new protocol the solvent was decanted into assay tubes and evaporated under filtered air in a 37°C water bath. (Should adequate sensitivity have been attained using evaporation under air, the use of costly nitrogen would be unnecessary).

To each tube the following were added:

. 500µl 1% ethanol assay buffer - both series (vortex and equilibration for 1 hour at room temperature)

. 100µl 1:3750 antiserum)
) Series 1
. 100µl 1:100 [^{3}H] melatonin)

. 100µl 1:5625 antiserum)
) Series 2
. 100µl 1:150 ^{3}H melatonin)

. 100µl assay buffer wash down - both series.

Total incubation volume = 800µl.

Following incubation for 20 hours at 4°C the bound and unbound fractions were separated by addition of 200µl 0.625% dextran-coated charcoal for 30 minutes at 4°C. The tubes were centrifuged (2000rpm for 6 minutes), and 750µl of the supernatant was transferred to scintillation vials and counted after 24 hours quenching.

Non-specific binding (NSB)

To determine NSB for both series three tubes containing one ml assay buffer and one ml borate buffer were extracted as described, and to each was added,

- 500µl 1% ethanol assay buffer,
- 100µl normal assay buffer,
- 100µl either 1:100 or 1:150 [^{3}H] melatonin, and
- 100µl assay buffer washdown.

These were incubated, separated and counted as for the standards.

Solvent blanks

To determine any interference in the assay from the solvents used in the extractions, for both series three tubes without the assay or borate buffers were extracted and incubated, separated and counted as for the standards.

Totals

To determine the total amount of tracer added to each series during incubation, three assay tubes containing

- 500µl 1% ethanol assay buffer,
- 100µl normal assay buffer,
- 100µl either 1:100 or 1:150 [^{3}H] melatonin, and
- 100µl normal assay buffer washdown, were not extracted

or separated, and 750µl was counted.

For both series the amount of tracer bound in the standards and blanks, less NSB, was expressed as a percentage of the Totals (B/T), and as a percentage of binding at the zero standard (B/Bo). The displacement curves for both series are shown in Figure 5.3-10 and the means and two

standard deviations from the means are given in Table 5.3-11. For both series the amount of binding of the tracer (B/T and B/Bo) decreased with increasing amount of standard melatonin, indicating competition between the [^{3}H] melatonin and melatonin for binding sites in the antiserum. According to the criterion used to establish assay sensitivity, both series give an assay sensitivity of 15.6pg/ml Table 5.3-11. These are consistent with that reported by Kennaway *et al.* (1982) of 20 ± 7pg/ml, indicating that evaporation of the solvent under air, instead of nitrogen, did not sufficiently reduce recovery of melatonin to alter assay sensitivity.

The maximum binding (B/T at zero standard) obtained in series 1 (41.9%) was less than that obtained for an antiserum dilution of 1:5000 in the non-extracted curve used for the antiserum titration procedure (56.04%). A higher binding for series 1 was expected, as the antiserum dilution was decreased to 1:3750 and the tracer dilution was not changed. This lower binding could have been attributed to either interference from the extraction procedure or the ethanol during incubation.

It was possible residual impurities of the solvents had interfered with the antibody/tracer complex by either (i) occupying antibody sites and so displacing the tracer, or (ii) decreasing the affinity of the antibody for the tracer by denaturing the antibody. If impurities had been displacing tracer, then binding in the blanks would be lower than that of the assay sensitivity. However as binding in the blanks of series 1 (B/Bo = 99.2%, B/T = 41.7%, Table 5.3-11 were higher than those obtained for that of the assay sensitivity (15.6pg/ml, B/Bo = 95.4%, B/T = 40.9%), the lower maximum binding cannot be

attributed to high blank values and therefore may have been due to denaturing of the antibody by the solvents or interference by the ethanol.

It was considered essential, therefore, that the melatonin standard curve was extracted along with samples in routine use of the assay. If it were not, this demonstrated decrease in binding following extraction and use of ethanol in the incubation medium would result in higher calculated levels of melatonin in samples by reference to the non-extracted standard curve.

The maximum binding obtained for series 2 (38%, Table 5.3-11) was less than that for series 1. However the slope of the displacement curve was steeper than that of series 1 (Figure 5.3-10), so was more able to distinguish between doses of standard than the curve generated by series 1. For example, in series 1 the range of B/Bo values between the 15.6 and 125pg standards (109.4 difference) is from 95.4 to 74.8 (20.6 difference). For series 2 this is from 95.1 to 65.0 (30.1 difference). A one percent change in B/Bo for series 1 therefore causes a 5.31pg change in calculated melatonin dose (109.4 divided by 20.6) but, in series 2, a one percent change in B/Bo causes a 3.63pg change in melatonin dose (109.4 divided by 30.1). However, as the lower amount of bound tracer in series 2 could have led to lower sensitivity resulting from counting error in subsequent assays, a slightly higher concentration of antiserum was considered necessary to increase binding of the tracer and so counting accuracy.

For subsequent assays, therefore, the melatonin standard curve and samples were extracted, the solvent evaporated under air, and the extracted crystalline melatonin dissolved in 1% ethanol assay buffer. To each tube was added:

- 100µl [^{3}H] melatonin (working dilution 1:150),
- 100µl Antiserum G280 (working dilution 1:5150, Table 5.3-9)
- 100µl assay buffer washdown, and following incubation

for 14 hours at 4^{o}C, the fractions were separated with charcoal as described and 750µl supernatant counted.

The displacement curves for subsequent assays (N=16) performed under these new conditions were found to be consistent, as demonstrated by the standard error at each standard (Figure 5.3-12, Table 5.3-13). Sensitivity of these assays was 20.5 $\pm$ 9.0pg/tube which is consistent with that reported by Kennaway *et al.* (1982) of 20.0 $\pm$ 7.0pg/ml.

(v) Assay procedure

This section is a synopsis of the assay procedure that was developed for routine use in the tammar.

(a) Extraction of lipids and melatonin

In 15ml glass extraction tubes (Quickfit) 1ml borate buffer was added to 1ml plasma sample or standard followed by 6ml petroleum spirit

(B.R. 60-80^{o}C). Lipid was extracted by rocking the tubes at 48 rocks per minute for 30 minutes on a Dynamax flask shaker. After centrifugation at 2000rpm for 6 minutes the solvent and lipid interface were carefully aspirated off.

The melatonin was extracted by addition of 6mls dichloromethane: hexane (1:1) and vigorous rocking (140 rocks per minute) for 30 minutes. Following separation of the aqueous and solvent phases, the aqueous phase was frozen in a solid Co_2/ethanol bath. The solvent was decanted into 13 x 100mm glass assay tubes and evaporated under filtered air in a 37^{o}C water bath.

(b) Incubation:

To each tube the following were added

- 500µl 1% ethanol assay buffer (vortex and equilibrate for 1 hour)
- 100µl antiserum G280 (working dilution 1:5150, Table 5.3-9)
- 100µl [^{3}H] melatonin (working dilution 1:150, section 5.2(iii)
- 100µl assay buffer washdown and vortex giving a total incubation volume of 800µl. The tubes were incubated overnight (14 hours) at 4^{o}C.

(c) Separation:

The unbound melatonin fraction was removed by addition of 200µl dextran coated charcoal (0.625%, section 5.2(v)), vortex, and incubation for 30 minutes at 4°C. After centrifugation (2000rpm for 6 minutes), 750µl of the supernatant containing bound radioligand was added to 10mls scintillation fluid (section 5.2(vi)) for counting.

(d) Calculations:

For each standard and sample, the amount of antibody bound (^{3}H) melatonin (B) minus non-specific binding (NSB), was expressed as a percentage of the amount bound in the zero standard (Bo) and of the total amount added (T). Following transformation of %B/Bo to LOGIT, where LOGIT = $\ln \frac{p}{100-p}$ ($\ln$ = natural logarithm, p = %B/Bo) and fitting a regression line to the LOGIT standard curve, the amount of melatonin in unknown plasma samples was determined by reference to the regression line.

Transformation to LOGIT was considered preferable to use of a B/Bo plot, as LOGIT allows a straight line function to be fitted using a regression equation, so taking into account the correlation coefficient of the whole bivariate function. This was more objective than plotting a B/Bo curve by freehand. To enable calculation of a regression equation, log dose (standards) had to be transformed to a linear scale, as LOGIT was linear. Figure 5.3-14 gives examples of a B/Bo and LOGIT plot obtained from values of the same standard curve (Table 5.3-15).

The calculated regression equation for this standard curve is $y = -0.025x + 3.233$ with a correlation coefficient (r) = -0.997. From the regression equation, estimates at X = 25.5 (15.6pg) and X = 178.5 (1000pg) were calculated and a linear function plotted between those points. The melatonin concentration in unknown samples was determined by reference to the LOGIT plot. For example, an unknown sample with a calculated LOGIT of 1.0 was determined to contain 95pg of melatonin (LOGIT radioimmunoassay sheets include finer graduations than those shown in Figure 5.3-14.

(e) Assay characteristics:

At a final dilution of 1:41,200 the antiserum bound 40.6 $\pm$ 3.5% (mean $\pm$ standard deviation, Table 5.3-13) of [^{3}H] melatonin (final dilution - 1:1,200). Non-specific binding of [^{3}H] melatonin was less than 3%. Plasma and solvent blanks were less than assay sensitivity except on four occasions where plasma blanks of 37.8 $\pm$ 22.2pg/ml (mean $\pm$ standard deviation) were recorded and corrected for. Assay sensitivity was 20.5 $\pm$ 9.0 pg/tube.

5.4 Assay validation for the tammar

(i) Parallelism of dose response curves

To ensure that normally occurring factors in tammar plasma did not interfere in the assay system, and that the endogenous hormone behaved identically to standard melatonin, it was necessary to demonstrate that dose response curves of endogenous and standard hormone in a plasma and buffer medium were parallel.

Two series of melatonin standards (3.9 to 1000pg/ml) in triplicate were set up in absolute ethanol, evaporated under air and resuspended in 1ml assay buffer or pinealectomized tammar plasma. A tammar pineal gland was homogenized in 2.2ml assay buffer, centrifuged and two 1ml aliquots of the supernatant added to extraction tubes. One ml borate buffer was added to all tubes which were then extracted and assayed as previously described.

The buffer and plasma standards were resuspended in 500µl 1% ethanol assay buffer and assayed as described. The solvent extracts of the pineal supernatant were pooled, evaporated and resuspended in 4ml 1% ethanol buffer. From this 10µl, 30µl, 50µl, 100µl, 200µl and 500µl aliquots were taken in triplicate, made up to 500µl with 1% ethanol buffer, and assayed.

The plasma standard and pineal extract displacement curves were parallel to the buffer standard displacement curve (Figure 5.4-1, Table 5.4-2), indicating that factors in tammar plasma and pineal tissue did not interfere in the assay, and that the endogenous hormone of pineal origin behaved similarly to the standard hormone.

Furthermore, the parallelism of the buffer and plasma curves, and the similar recoveries of melatonin from these media (Table 5.3-8), indicated a buffer standard curve could be used in routine assays.

(ii) Recovery of exogenous melatonin

To ensure that the assay was accurately measuring the melatonin concentration in plasma samples it was necessary to demonstrate correct recovery of exogenous melatonin. To achieve this, increasing amounts of melatonin were added to charcoal stripped plasma, and the concentration in each sample was compared after assay with the amount added. As the treatment with charcoal renders the plasma hormone free, the melatonin measured following assay should equal the amount of hormone added.

Melatonin standards in ethanol were added to a series of extraction tubes in triplicate (Table 5.4-3). The ethanol was evaporated under filtered air and the melatonin in each tube resuspended in 1ml tammar plasma from a charcoal stripped pool. One ml borate buffer was added to each and the samples were extracted and assayed according to the procedure outlined in 5.3(v). A buffer standard curve was also set up in triplicate, extracted and assayed. The melatonin concentration of each plasma sample was determined by reference to the buffer standard curve.

The amount of melatonin added to charcoal stripped tammar plasma was similar to that measured following assay, within the range of 50-1000pg/ml (Table 5.4-4). The regression equation for melatonin added (x) plotted against melatonin measured (y) was $y = 0.975x + 4.923$ with a correlation coefficient (r) = 0.999 (Figure 5.4-5).

These results showed that the assay could accurately measure melatonin concentrations in tammar plasma which also indicated a lack of interference in this assay from non-hormonal constituents of the plasma.

(iii) Intra- and interassay variation

To determine the variation in melatonin measurement within and between assays, aliquots of a sheep plasma pool containing both endogenous and exogenous melatonin were assayed in replicate in separate assays (n = 15) and in the same assay (n = 4). The intra- and interassay coefficients of variation (c.f. = $\frac{s}{\bar{x}} \times \frac{100}{1}$) were 4.5 - 10.5% and 16.7% respectively (Table 5.4-6), which compare favourably with those reported by Kennaway *et al.* (1982), of less than 10% and 20%.

(iv) Melatonin levels in pinealectomized tammars

To demonstrate that measured levels of the hormone were consistent with the physiology of the hormone, the circadian profiles of plasma melatonin were determined before and after extirpation of the pineal gland. Pinealectomy abolished the nocturnal rise of plasma melatonin, which is consistent with findings in other species. This aspect has already been considered in more detail in Chapter 3.

Table 5.3-1: The amount of assay buffer added to stock antiserum (1:25) to obtain different titrations of the antiserum.

Series	Antiserum Stock (1:25)	+	Assay Buffer	=	Working Dilution	Final Assay Dilution
1	50µl		6ml		1:3000	1:24,000
2	50µl		10ml		1:5000	1:40,000
3	50µl		14ml		1:7000	1:56,000

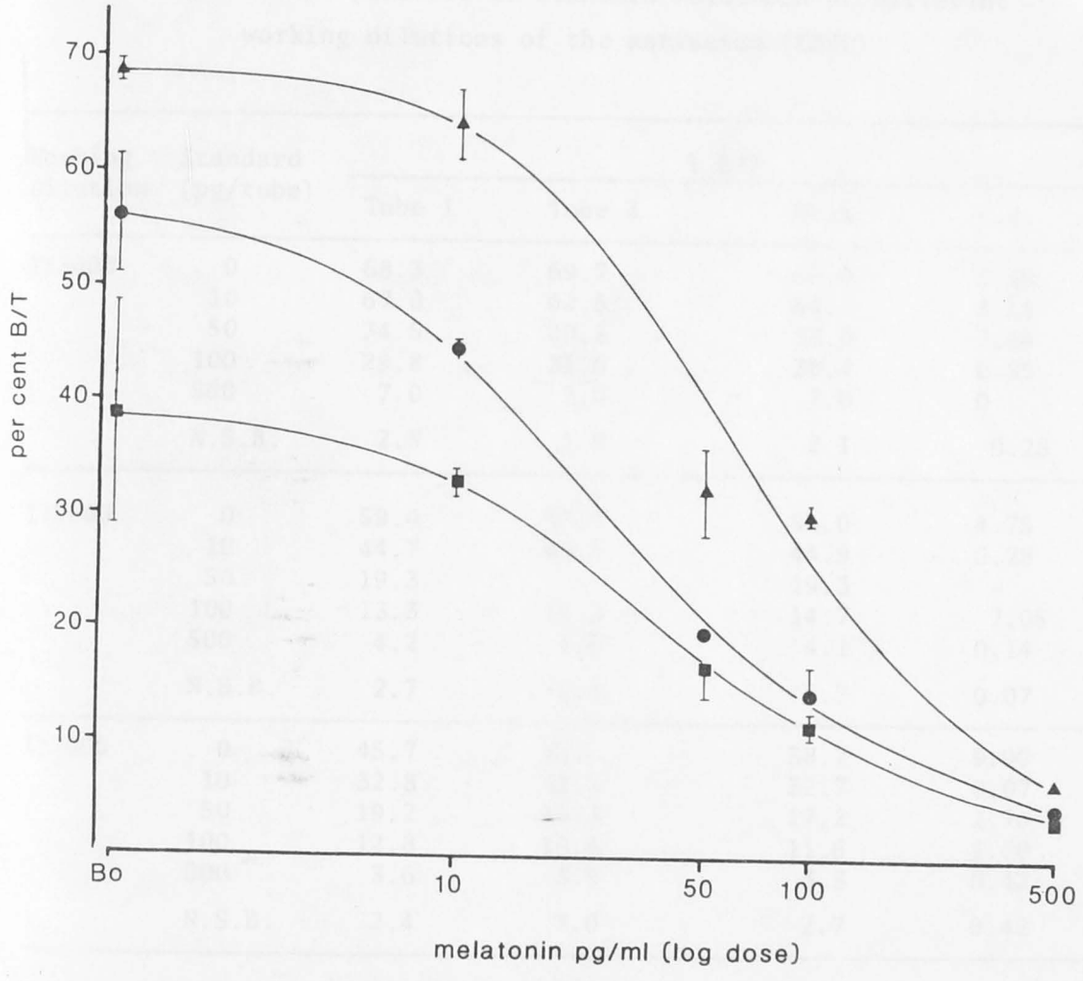


Figure 5.3-2: The displacement curves obtained in the presence of standard melatonin for working dilutions of antiserum at 1:3000 (▲), 1:5000 (●) and 1:7000 (■). Values given are the mean ± standard deviation.

Table 5.3-3: The percent binding of [^{3}H] melatonin (B/T) obtained in the presence of standard melatonin at different working dilutions of the antiserum (G280).

Working Dilution	Standard (pg/tube)	% B/T			
		Tube 1	Tube 2	Mean	s.d.
1:3000	0	68.3	69.7	69.0	0.99
	10	67.0	62.5	64.7	3.18
	50	34.5	29.5	32.0	3.54
	100	29.8	31.0	30.4	0.85
	500	7.0	7.0	7.0	0
	N.S.B.	2.3	1.9	2.1	0.28
1:5000	0	59.4	52.7	56.0	4.75
	10	44.7	45.1	44.9	0.28
	50	19.3	-	19.3	-
	100	13.3	16.2	14.7	7.05
	500	4.2	4.0	4.1	0.14
	N.S.B.	2.7	2.6	2.7	0.07
1:7000	0	45.7	31.7	38.7	9.90
	10	32.8	32.7	32.7	0.07
	50	19.2	15.3	17.2	2.76
	100	12.8	10.4	11.6	1.70
	500	3.6	3.0	3.3	0.42
	N.S.B.	2.4	3.0	2.7	0.42

Table 5.3-4: The original and new protocols for obtaining melatonin standards in the range of 0-1000pg.

ORIGINAL PROTOCOL

Stock Standard A - 1mg/ml in ethanol
B - 10µg/ml in ethanol
C - 100ng/ml in assay buffer
D - 10ng/ml in assay buffer
E - 1ng/ml in assay buffer

Standard (pg/500µl)	= Assay Buffer +	Stock Standard
0	500µl	0
10	490µl	10µl E
20	480µl	20µl E
30	470µl	30µl E
50	450µl	50µl E
70	430µl	70µl E
100	400µl	100µl E
200	480µl	20µl D
300	470µl	30µl D
500	450µl	50µl D
1000	400µl	100µl D

NEW PROTOCOL (Double dilution)

Stock Standard A - 2mg/ml in ethanol
B - 20µg/ml in ethanol
C - 200ng/ml in assay buffer
D - 20ng/ml in assay buffer

Standard (pg/500µl)	= Assay Buffer	+ Standard
1000	9ml	1ml D (stock)
500	2ml	2ml (1000pg/500µl)
250	2ml	2ml (500pg/500µl)
125	2ml	2ml (250pg/500µl)
62.5	2ml	2ml (125pg/500µl)
31.3	2ml	2ml (62.5pg/500µl)
15.6	2ml	2ml (31.3pg/500µl)
7.8	2ml	2ml (15.6pg/500µl)
3.9	2ml	2ml (7.8pg/500µl)
0	2ml	-

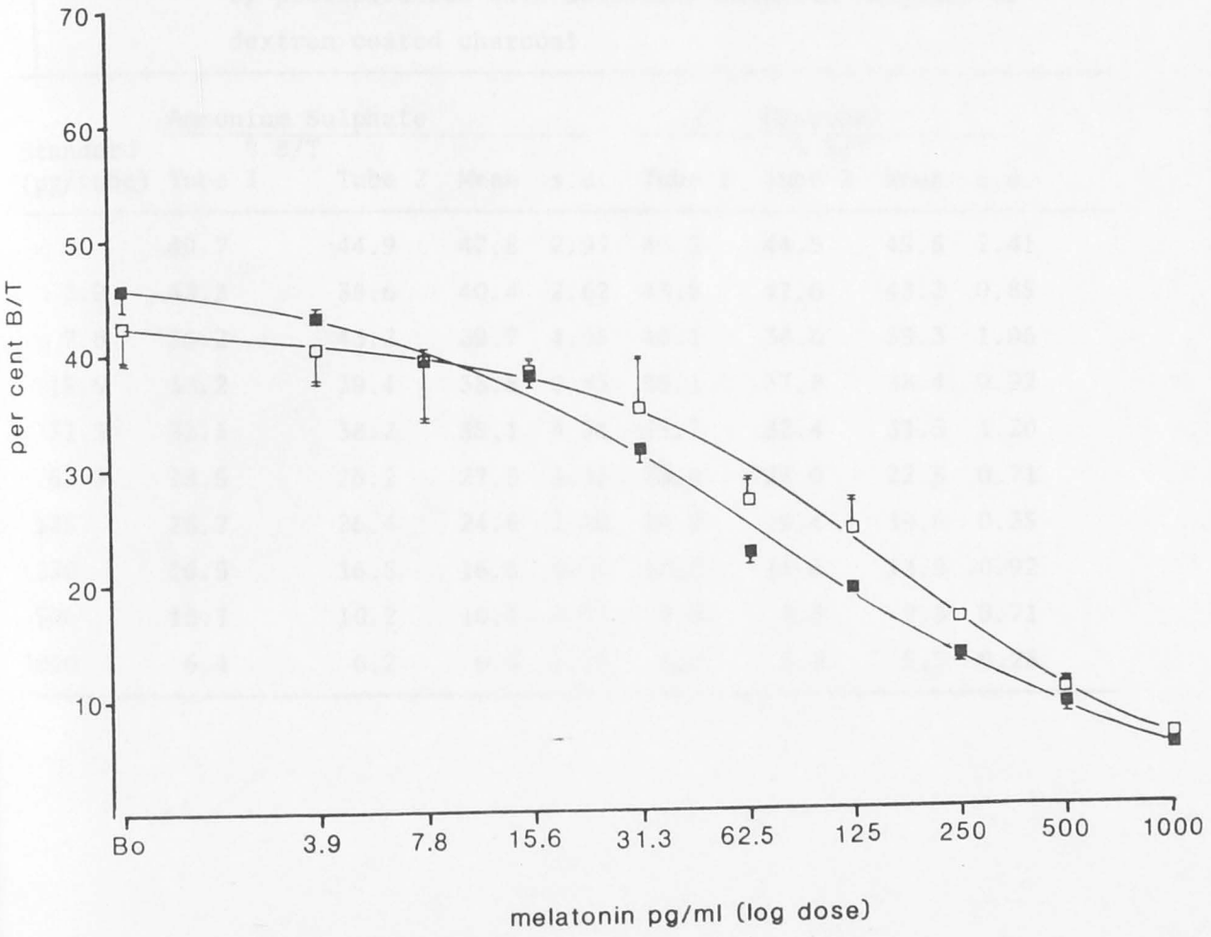


Figure 5.3-5: The displacement curves obtained for two series of melatonin standards following removal of the unbound fractions by precipitation with saturated ammonium sulphate (□) or dextran coated charcoal (■). Values given are the mean ± standard deviation.

Table 5.3-6: Comparison of percent binding of [^{3}H] melatonin (B/T) for two series of melatonin standard curves in which the unbound fraction of [^{3}H] melatonin was removed by precipitation with saturated ammonium sulphate or dextran coated charcoal.

Standard (pg/tube)	Ammonium Sulphate % B/T				Charcoal % B/T			
	Tube 1	Tube 2	Mean	s.d.	Tube 1	Tube 2	Mean	s.d.
0	40.7	44.9	42.8	2.97	46.5	44.5	45.5	1.41
3.9	42.3	38.6	40.4	2.62	43.8	42.6	43.2	0.85
7.8	36.2	43.2	39.7	4.95	40.1	38.6	39.3	1.06
15.6	38.2	39.4	38.8	0.85	39.1	37.8	38.4	0.92
31.3	32.1	38.2	35.1	4.31	30.7	32.4	31.5	1.20
62.5	28.5	26.2	27.3	1.63	23.0	22.0	22.5	0.71
125	23.2	26.4	24.8	2.26	19.9	19.4	19.6	0.35
250	16.5	16.5	16.5	0.00	13.3	14.6	13.9	0.92
500	10.1	10.2	10.1	0.07	9.8	8.8	9.3	0.71
1000	6.4	6.2	6.3	0.15	5.7	5.3	5.5	0.28

Table 5.3-7 : The efficiency of extraction of [^{3}H] melatonin from a buffer and tammar plasma medium using the original protocol. Values given are the amount recovered as a percentage of the amount added.

Replicate	Extraction Efficiency (%)	
	Buffer	Plasma
1	58.8	55.7
2	61.2	55.7
3	75.8	57.9
4	63.6	53.8
5	54.5	53.1
6	63.3	49.7
7	64.0	56.0
8	61.1	55.8
9	65.5	55.9
10	64.0	48.3
Mean	63.2	54.2
s.d.	5.5	3.0

Table 5.3-8: The efficiency of extraction of [^{3}H] melatonin from a buffer and charcoal stripped tammar plasma medium in the presence of unlabelled melatonin. Values given are expressed as a percentage of the amount added.

Extraction Medium	Melatonin Concentration (pg/ml)	Extraction efficiency (%) Tube number 1	2	3	mean ± s.e.m.	
Buffer	0	63	87	84	78	7.55
	31.3	80	84	87	87.67	2.03
	125	84	84	84	84	0
	500	81	80	70	77	3.51
			mean ± s.e.m.		80.67 ±	1.84
Plasma	0	64	80	86	76.67	6.57
	31.3	86	86	86	86	0
	125	87	88	88	87.67	0.33
	500	80	80	66	75.33	4.67
			mean ± s.e.m.		81.42 ±	3.16

Table 5.3-9: The amount of buffer added to stock antiserum (1:25) and stock [^{3}H]melatonin (1:50) to obtain various ratios of each.

ANTISERUM					TRACER				
Antiserum Stock (1:25)	+	Assay Buffer	= Working Dilution	Final Assay Dilution	Tracer Stock (1:50)	+	Assay Buffer	= Working Dilution	Final Assay Dilution
50µl		7.5mls	1:3750	1:30,000	50µl		5mls	1:100	1:800
50µl		11.25mls	1:5625	1:45,000	50µl		7.5mls	1:150	1:1200
50µl		10.30mls	1:5150	1:41,200	50µl		7.5mls	1:150	1:1200

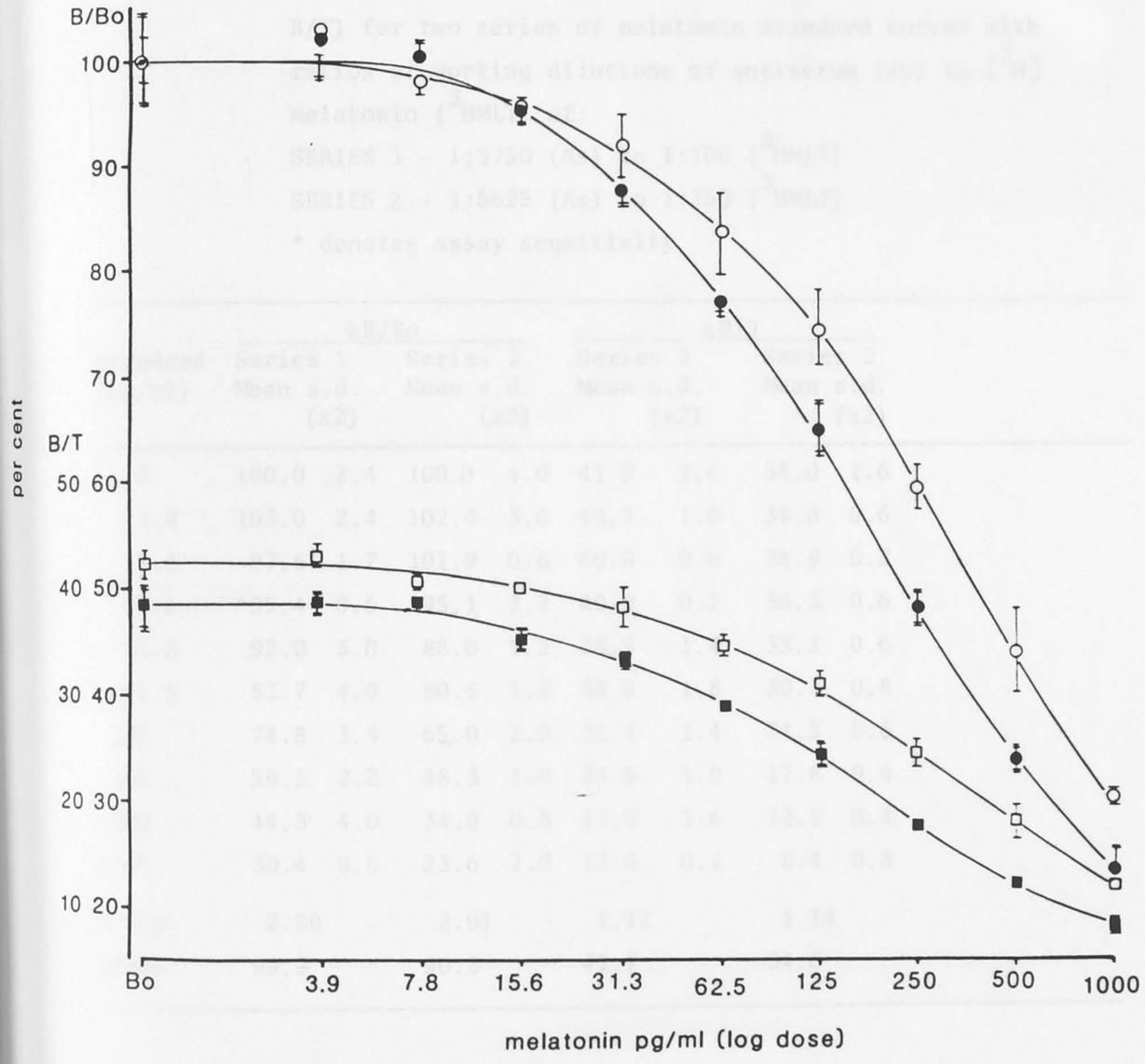


Figure 5.3-10: The displacement curves obtained for ratios of working dilutions of antiserum (As) to radioligand ([^{3}H] MLT) at:

- 1:100 As to 1:3750 [^{3}H]MLT, (B/Bo = ○, B/T =□)
- 1.:150 As to 1:5625 [^{3}H]MLT, (B/Bo =● , B/T = ■)

Values given are the mean ± standard deviation.

Table 5.3-11: Comparison of percent binding of [^{3}H] melatonin (B/Bo and B/T) for two series of melatonin standard curves with ratios of working dilutions of antiserum (As) to [^{3}H] melatonin (^{3}HMLT) of:
SERIES 1 - 1:3750 (As) to 1:100 (^{3}HMLT)
SERIES 2 - 1:5625 (As) to 1:150 (^{3}HMLT)
* denotes assay sensitivity

Standard (pg/ml)	%B/Bo Series 1 Mean	s.d. (x2)	%B/Bo Series 2 Mean	s.d. (x2)	%B/T Series 1 Mean	s.d. (x2)	%B/T Series 2 Mean	s.d. (x2)
0	100.0	2.4	100.0	4.0	41.9	1.0	38.0	1.6
3.9	103.0	2.4	102.4	3.6	43.2	1.0	38.8	0.6
7.8	97.6	1.2	101.9	0.6	40.9	0.6	38.8	0.2
15.6	*95.4	0.6	*95.1	1.2	40.0	0.2	36.3	0.6
31.3	92.0	3.0	88.0	1.2	38.5	1.4	33.3	0.6
62.5	83.7	4.0	80.5	1.2	34.9	1.8	30.3	0.8
125	74.8	3.4	65.0	2.0	31.1	1.4	24.3	0.6
250	59.5	2.2	48.3	1.0	24.5	1.0	17.8	0.4
500	44.3	4.0	34.0	0.8	17.9	1.6	12.2	0.4
1000	30.4	0.6	23.6	2.0	12.0	0.2	8.1	0.8
N.S.B.	2.59		2.91		1.12		1.14	
Blank	99.2		90.8		41.7		34.0	

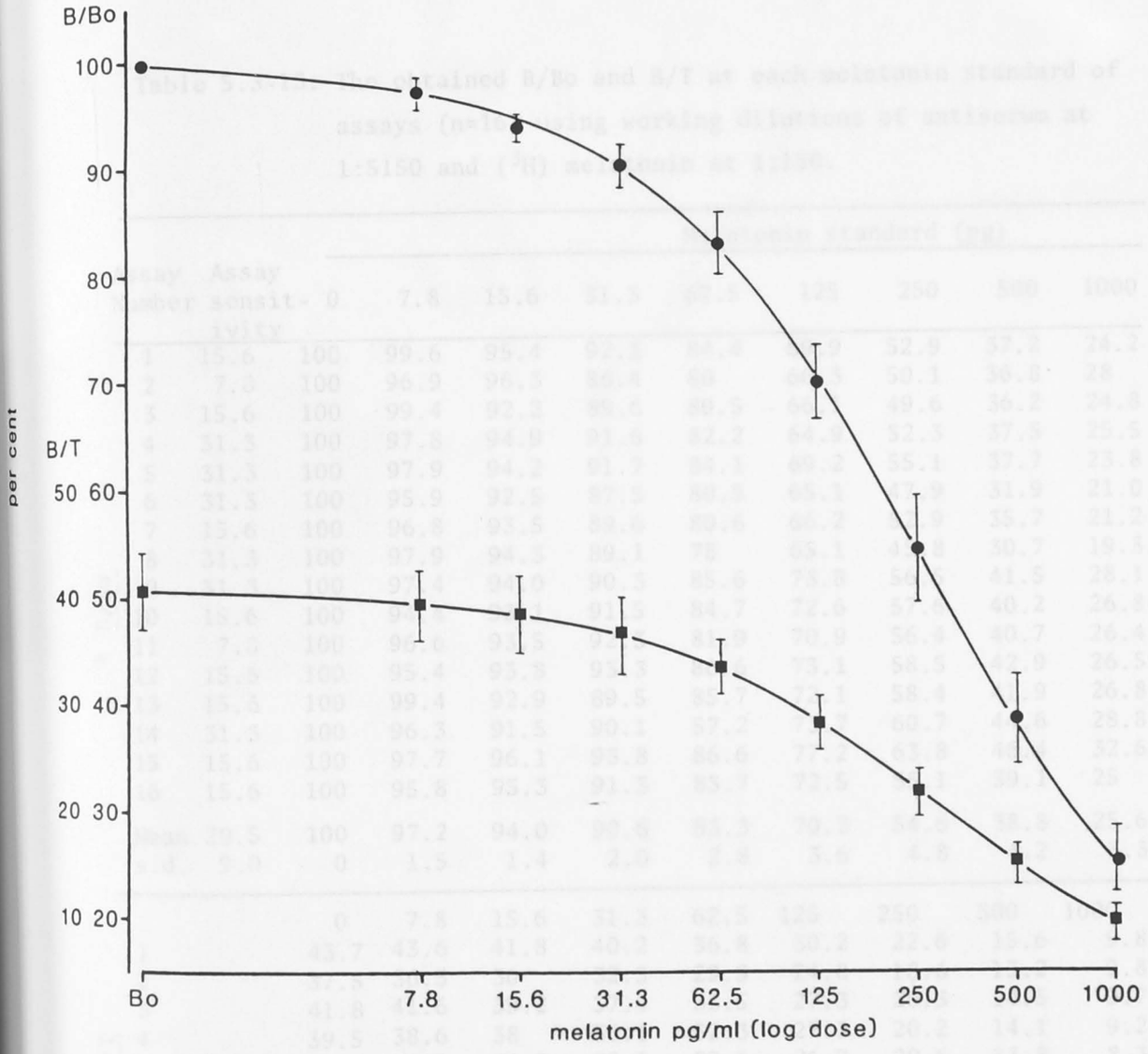


Figure 5.3-12: The displacement curves obtained for 16 assays using working dilutions of antiserum at 1:5150 and [^{3}H] melatonin at 1:150. Values given are the mean ± standard deviation for B/Bo (●) and B/T (■) obtained from all assays.

Table 5.3-13: The obtained B/Bo and B/T at each melatonin standard of assays (n=16) using working dilutions of antiserum at 1:5150 and (^{3}H) melatonin at 1:150.

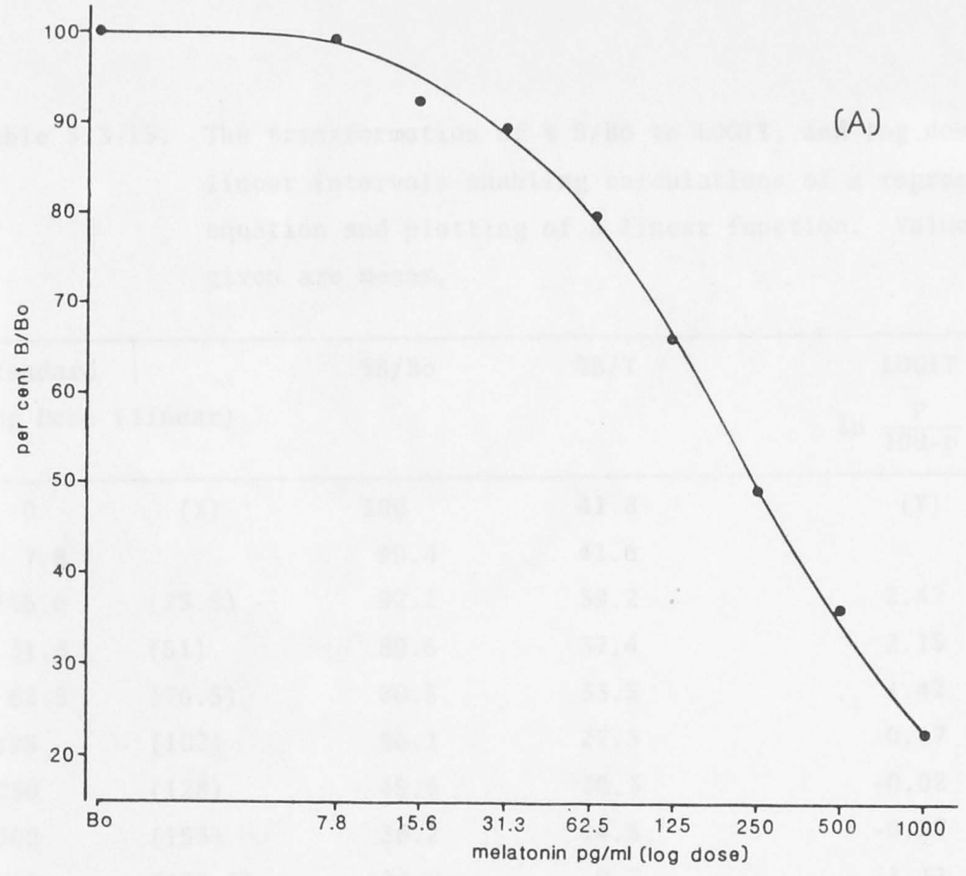
			Melatonin standard (pg)							
Assay Number	Assay sensitivity	0	7.8	15.6	31.3	62.5	125	250	500	1000
1	15.6	100	99.6	95.4	92.1	84.4	69.9	52.9	37.2	24.2
2	7.8	100	96.9	96.3	86.4	80	66.3	50.1	36.8	28
3	15.6	100	99.4	92.2	89.6	80.5	66.1	49.6	36.2	24.8
4	31.3	100	97.8	94.9	91.6	82.2	64.9	52.3	37.5	25.5
5	31.3	100	97.9	94.2	91.7	84.1	69.2	55.1	37.7	23.8
6	31.3	100	95.9	92.5	87.5	80.5	65.1	47.9	31.9	21.0
7	15.6	100	96.8	93.5	89.6	80.6	66.2	52.9	35.7	21.2
8	31.3	100	97.9	94.3	89.1	78	65.1	45.8	30.7	19.3
9	31.3	100	97.4	94.0	90.3	85.6	73.8	56.5	41.5	28.1
10	15.6	100	94.4	93.1	91.5	84.7	72.6	57.6	40.2	26.8
11	7.8	100	96.6	93.5	92.3	81.9	70.9	56.4	40.7	26.4
12	15.6	100	95.4	93.8	93.3	86.6	73.1	58.5	42.9	26.5
13	15.6	100	99.4	92.9	89.5	85.7	72.1	58.4	41.9	26.8
14	31.3	100	96.3	91.5	90.1	57.2	73.7	60.7	44.6	28.8
15	15.6	100	97.7	96.1	93.8	86.6	77.2	63.8	46.4	32.6
16	15.6	100	95.8	95.3	91.3	83.7	72.5	55.1	39.1	25
Mean	20.5	100	97.2	94.0	90.6	83.3	70.2	54.6	38.8	25.6
s.d.	9.0	0	1.5	1.4	2.0	2.8	3.6	4.8	4.2	3.3

% B/Bo

	0	7.8	15.6	31.3	62.5	125	250	500	1000
1	43.7	43.6	41.8	40.2	36.8	30.2	22.6	15.6	9.8
2	37.5	36.3	36	32.3	29.9	24.8	18.6	13.2	9.8
3	41.8	41.6	39.2	37.4	33.5	27.3	20.3	14.5	9.7
4	39.5	38.6	38	36.1	32.3	27.3	20.2	14.1	9.2
5	38.3	37.5	36.2	35.1	32.1	26.2	20.6	13.8	8.3
6	44.8	42.9	41.4	39.0	35.9	28.8	21.0	13.6	8.7
7	40.9	39.6	38.6	36.6	32.7	26.7	21.1	13.8	7.8
8	46.2	45.2	43.5	41.1	35.8	29.7	20.6	13.4	8.0
9	33.8	32.9	31.4	30.4	28.8	24.6	18.6	13.4	8.7
10	44.8	42.3	41.7	41.0	37.9	32.3	25.5	17.5	11.3
11	41.5	40.1	38.8	38.2	33.8	29.1	23	16.3	10.2
12	41.9	39.9	39.2	39	36.2	30.4	24.1	17.5	10.5
13	35.6	35.3	33.0	31.8	30.4	25.4	20.5	14.5	9.0
14	36.5	35.1	33.3	32.8	31.7	26.7	21.86	15.8	9.9
15	41.8	40.8	40.1	39.2	36.1	32.1	26.3	18.8	12.9
16	41.3	39.5	39.4	37.6	34.4	29.7	22.4	15.6	9.7
Mean	40.6	39.5	38.2	36.7	33.6	28.2	21.7	15.1	9.6
s.d.	3.5	3.4	3.4	3.4	2.7	2.4	2.2	1.7	1.3

% B/T

Figure 5.3-14: Displacement curves from the same melatonin standards expressed as B/Bo (A) and LOGIT (B).


(A)
per cent B/Bo
100
90
80
70
60
50
40
30
20
Bo
7.8
15.6
31.3
62.5
125
250
500
1000
melatonin pg/ml (log dose)

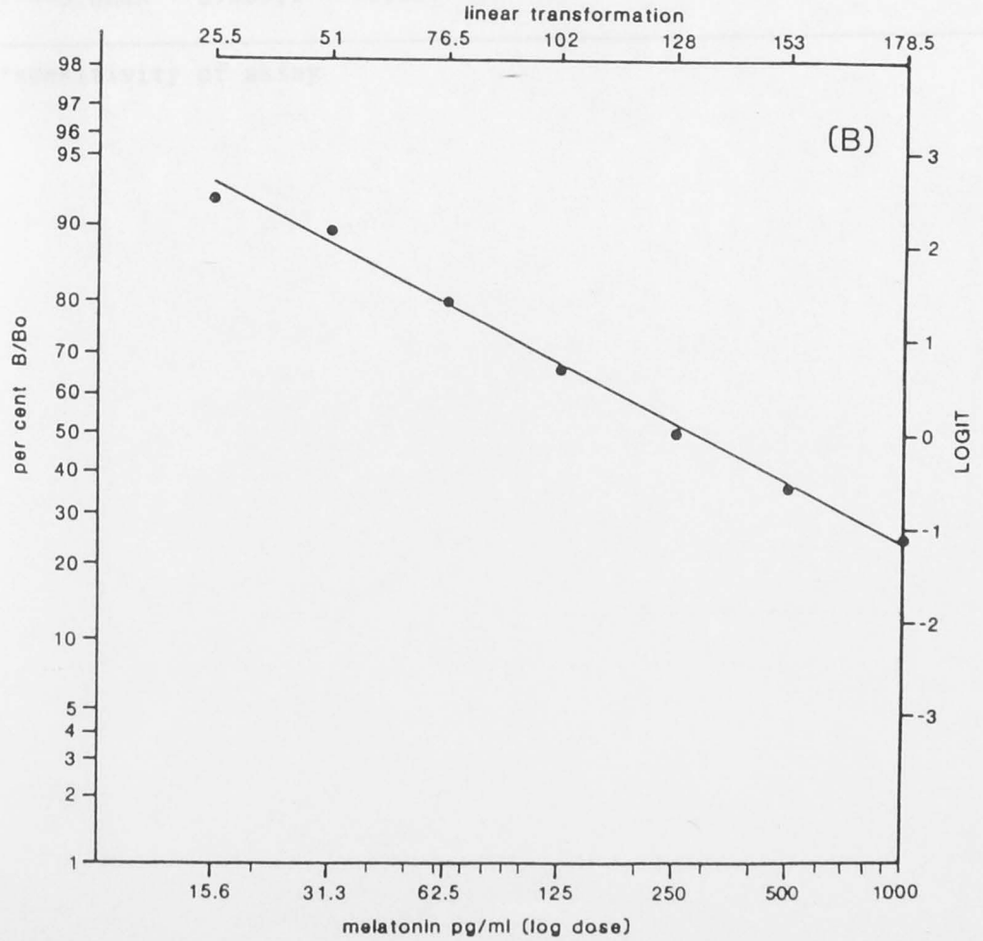

linear transformation
25.5
51
76.5
102
128
153
178.5
(B)
per cent B/Bo
LOGIT
98
97
96
95
90
80
70
60
50
40
30
20
10
5
4
3
2
1
3
2
1
0
-1
-2
-3
15.6
31.3
62.5
125
250
500
1000
melatonin pg/ml (log dose)

Table 5.3-15: The transformation of % B/Bo to LOGIT, and log dose to linear intervals enabling calculations of a regression equation and plotting of a linear function. Values given are means.

Standard Log Dose	(linear)	%B/Bo	%B/T	LOGIT $\ln \frac{P}{100-p}$
0	(X)	100	41.8	(Y)
7.8		99.4	41.6	
*15.6	(25.5)	92.2	39.2	2.47
31.3	(51)	89.6	37.4	2.15
62.5	(76.5)	80.5	33.5	1.42
125	(102)	66.1	27.3	0.67
250	(128)	49.6	20.3	-0.02
500	(153)	36.2	14.5	-0.57
1000	(178.5)	24.8	9.7	-1.11

$y = -0.025x + 3.233, r = -0.997$

*sensitivity of assay

Figure 5.4-1: Dose response curves for melatonin standard added to buffer (●) and pinealectomized tammar plasma (○), and for decreasing dilution of tammar pineal homogenate (▲). B/Bo (——), B/T (----).

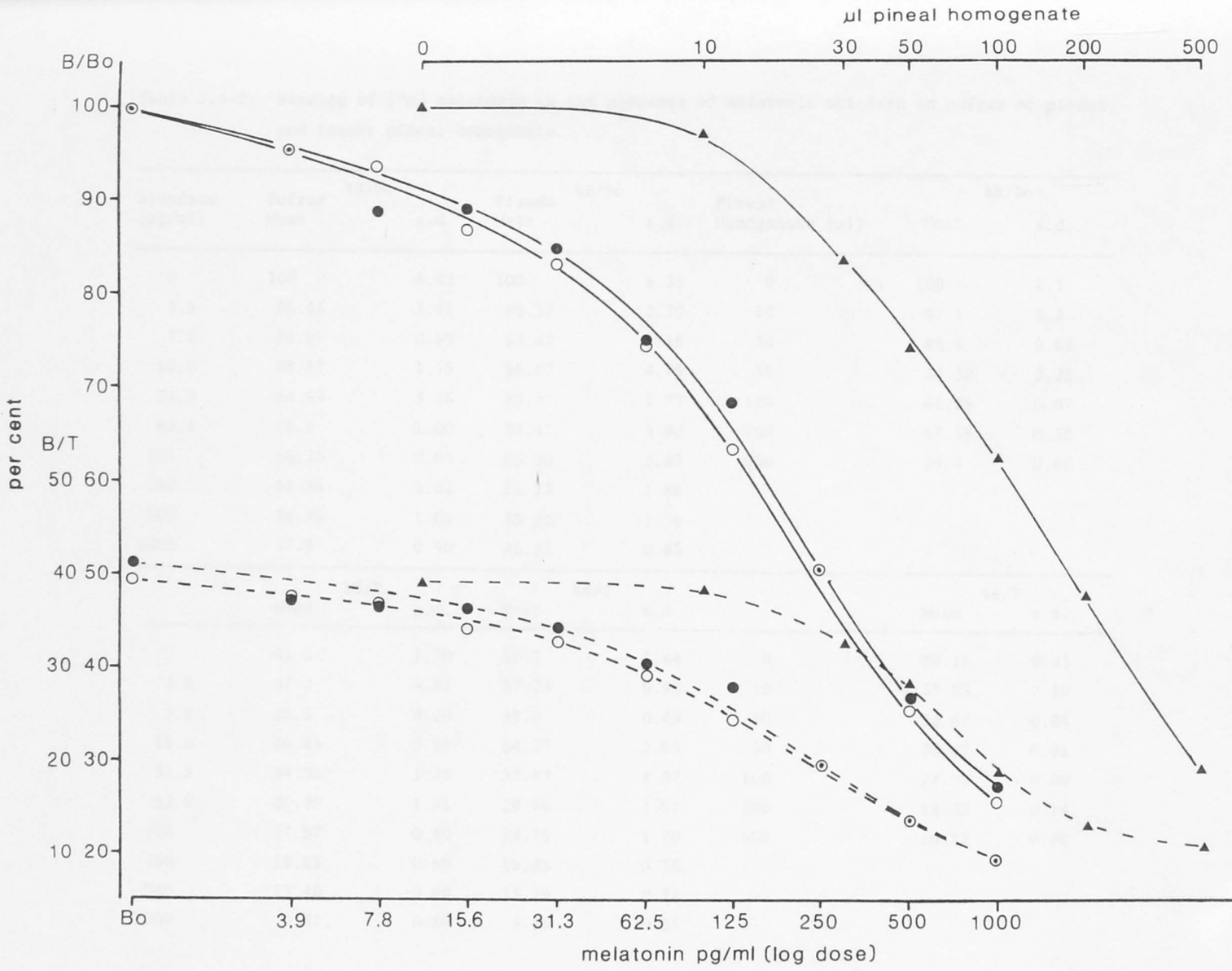

µl pineal homogenate
0
10
30
50
100
200
500
B/Bo
100
90
80
70
B/T
50 60
40 50
30 40
20 30
10 20
per cent
Bo
3.9
7.8
15.6
31.3
62.5
125
250
500
1000
melatonin pg/ml (log dose)

Table 5.4-2: Binding of [^{3}H] melatonin in the presence of melatonin standard in buffer or plasma, and tammar pineal homogenate.

Standard (pg/ml)	Buffer %B/BO Mean	s.d.	Plasma %B/Bo Mean	s.d.	Pineal Homogenate (µl)	%B/Bo Mean	s.d.
0	100	4.02	100	4.38	0	100	2.1
3.9	96.43	2.41	95.17	2.20	10	97.1	5.3
7.8	88.97	0.55	93.47	1.16	30	83.9	2.09
15.6	88.87	1.15	86.67	4.79	50	74.39	2.28
31.3	84.59	3.05	83.2	2.77	100	62.55	0.07
62.5	75.1	1.00	74.47	3.92	200	47.55	0.35
125	68.73	0.91	63.30	2.83	500	29.4	0.60
250	51.36	1.62	51.23	1.86			
500	36.75	1.63	35.23	1.76			
1000	27.4	0.50	25.63	0.83			
	%B/T Mean	s.d.	%B/T Mean	s.d.		%B/T Mean	s.d.
0	41.3	1.70	39.7	1.84	0	39.15	0.83
3.9	37.2	0.92	37.73	0.90	10	37.93	2.10
7.8	36.5	0.20	37.0	0.49	30	32.67	0.84
15.6	36.43	0.50	34.27	1.93	50	28.87	0.91
31.3	34.55	1.31	32.87	1.07	100	24.00	0.00
62.5	30.40	1.41	29.30	1.61	200	18.00	0.14
125	27.57	0.40	24.75	1.20	500	10.72	0.20
250	19.91	0.69	19.83	0.78			
500	13.48	0.69	13.29	0.72			
1000	9.31	0.30	9.38	0.34			

Table 5.4-3: Aliquots of melatonin in ethanol (1000pg/ml) added to charcoal stripped tammar plasma to determine recovery of the exogenous melatonin after assay.

Melatonin Added (pg/ml)	Aliquots of Melatonin in ethanol (1000pg/ml)	Tammar plasma (charcoal stripped)
0	0	1ml
50	50µl	1ml
75	75µl	1ml
100	100µl	1ml
200	200µl	1ml
500	500µl	1ml
750	750µl	1ml
1000	1000µl	1ml

Table 5.4-4: The amount of melatonin determined following assay of charcoal stripped tammar plasma containing added melatonin.

Melatonin Added (pg/ml)	Melatonin measured (pg/ml)				
	Tube 1	Tube 2	Tube 3	Mean	s.d.
0	<31.3	<31.3	<31.3	<31.3	
50	42	47	51	46.7	4.5
75	70	79	81	75.3	5.5
100	105	113	94	104.4	9.5
200	220	209	193	207.3	13.6
500	490	500	505	498.3	7.6
750	720	705	770	731.7	34.0
1000	950	990	1000+	980.0	26.5

$y = 0.975x + 4.923$

$r = 0.999$.

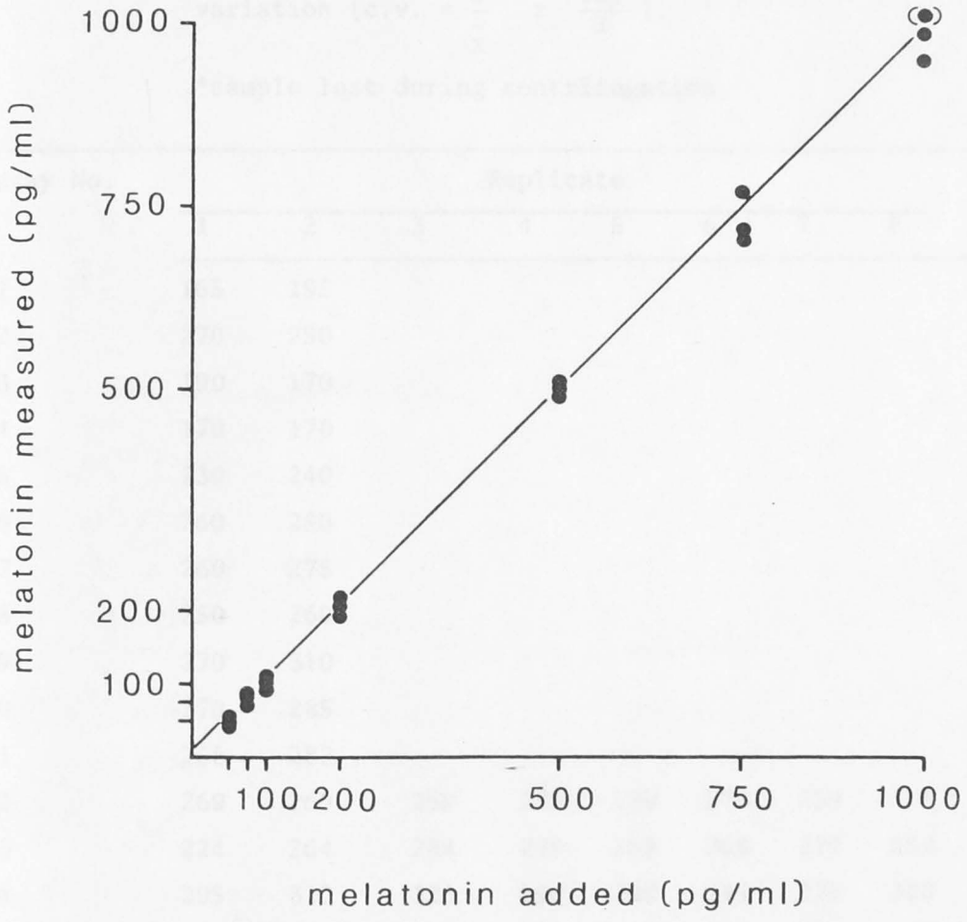


Figure 5.4-5: The amount of melatonin determined following assay plotted against the actual amount added. The regression equation is y = 0.975x + 4.923, with r = 0.999. ( ) sample > 1000pg/ml.

Table 5.4-6: The amount of melatonin (pg/ml) determined in aliquots of the same plasma pool in separate assays, providing (a) intra-assay and (b) inter-assay coefficients of variation (c.v. = $\frac{s}{\bar{x}} \times \frac{100}{1}$).

*sample lost during centrifugation.

Assay No.	Replicate								
	1	2	3	4	5	6	7	8	Mean
1	165	195							180
2	270	250							260
3	190	170							180
4	170	170							170
5	230	240							235
6	260	280							270
7	260	275							267
8	250	260							255
9	270	310							290
10	270	285							277
11	256	282							269
12	269	269	269	289	239	279	259	*	268
13	224	264	284	279	259	269	279	259	265
14	295	310	320	290	290	315	225	320	295
15	205	205	220	210	205	220	205	230	213
Assays 1 - 15								Mean	246.33
								s.d.	41.20
								c.v. (b) =	16.73

	Mean	s.d.	c.v.(a)
Assay 12	268	15.74	5.87
13	265	18.98	7.16
14	296	31.22	10.55
15	213	9.64	4.53

CHAPTER SIX

THE EFFECTS OF PINEALECTOMY AND GANGLIONECTOMY ON SEASONAL REPRODUCTION

General Introduction

6.1 Pinealectomy in seasonal quiescence (October)

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

6.2 Comparison of pinealectomy and ganglionectomy

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

6.3 Pinealectomy in April and July

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

6.4 Pinealectomy and the response to a stimulatory photoregimen

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

6.5 The effect of pinealectomy or ganglionectomy on seasonality in the year after surgery

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

THE EFFECTS OF PINEALECTOMY AND GANGLIONECTOMY ON SEASONAL REPRODUCTION

General Introduction

As discussed in Chapter 1, the pineal gland has been shown to be the neuroendocrine gland responsible for regulating the annual breeding cycle of a variety of mammals by providing seasonal photoperiod information. The demonstrated photosensitivity of the tammar (Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983), and the involvement of the superior cervical ganglia in the initiation of seasonal quiescence (Renfree *et al.* 1981), suggested that the pineal may also be involved in regulating the breeding cycle of this species. This chapter outlines a series of studies that examined the involvement of the pineal in seasonal breeding of the tammar by removal or denervation of the gland.

To illustrate whether pinealectomy or superior cervical ganglionectomy (ganglionectomy) affected the breeding cycle, it is necessary to recapitulate on features of the breeding cycle, and specify the endpoints used in these studies.

The first births of the breeding season are recorded from about January 14 to February 16. However, the breeding season usually extends to June as removal of the pouch young (RPY) up to June reactivates the CL and, if present, the associated blastocyst. Birth and/or oestrus occur 26 - 28 days after RPY. After June, RPY is ineffective and the animals are said to be in seasonal quiescence. As January 14

is the earliest birth date recorded in a limited number of years (Tyndale-Biscoe, *pers. comm.*) some animals may normally give birth before this time. To allow for this variation, in the experiments that follow, seasonal quiescence was arbitrarily assumed to have been abolished by pinealectomy or ganglionectomy if a birth/oestrus was recorded between June 22 (winter solstice) and December 22 (summer solstice). To detect birth/oestrus, regular checks, from one to three times a week, were usually made. For intervals longer than this, sometimes monthly, the date of birth was estimated from the pouch young's head length by reference to a growth curve for the tammar (Poole and Carpenter, unpub.).

6.1 Pinealectomy in seasonal quiescence (October).

(i) Introduction

Superior cervical ganglionectomy performed during lactational quiescence (in March to May) abolished seasonal quiescence (Renfree *et al.* 1981). These authors suggested that abolition of the circadian melatonin profile may have suppressed the elevated levels of prolactin associated with seasonal quiescence (Renfree *et al.* 1981), so removing the inhibitory influence of prolactin on the CL (Tyndale-Biscoe and Hawkins, 1977).

Should such a direct relationship between the pineal, prolactin and the corpus luteum exist, pinealectomy (PINX) during seasonal quiescence would prevent or depress the seasonal rise in plasma prolactin, allowing reactivation of the corpus luteum, and so abolition of seasonal quiescence. The present experiment was designed to test these predictions and determine any other changes in normal reproductive function following pinealectomy.

(ii) Materials and methods

Twelve female tammars that had their pouch young removed between June 7 and August 29, 1981 and were seasonally quiescent, were either pinealectomized (N = 6) or sham-operated (N = 6), between October 12 - 20, 1981. Their body weights ranged from 4.30 to 5.7kg. Following

recovery in the post-operative pens they were returned to outside yards by October 21 with sexually mature male tammars.

To monitor the circadian profiles of plasma melatonin and prolactin four-hourly blood samples over a 24 hour period were taken 20 - 28 days before, and 29 - 37 days after surgery (see Chapter 3.8-(i)). Of the tammars bled pre-operatively, one (No. 547) gave birth in October before surgery (so was excluded from the experiment) and another three died shortly after surgery (see Table 3.7-1). These animals were replaced with four females also in seasonal quiescence (Nos. 4700, 4772, 4950 and 4954). The pre- and post-operative profiles of plasma melatonin and statistical analyses of these have already been presented in Chapter 3.8(ii) and Appendices A.1 and A.2.

Blood samples for progesterone and prolactin were taken on the day of surgery, and then at weekly intervals, between 08.00hrs and 12.00hrs until detection of birth or oestrus, and again at autopsy in February, 1982. Plasma progesterone and prolactin were measured by RIA (Sernia, Hinds and Tyndale-Biscoe, 1980; Hinds and Tyndale-Biscoe, 1982b) by Dr. L. Hinds of the C.S.I.R.O. Division of Wildlife and Rangelands Research, Canberra. Regular checks were made to detect birth/oestrus and pouch condition.

All tammars were sacrificed by barbiturate overdose (Surital) on February 25 or 26, 1982, and the urogenital system was dissected out to record ovarian and uterine weights, corpora lutea weights and locations, the location of corpora albicantia, and to recover ova or embryos from

the uteri (see Chapter 2.3-(ii)). Histological examination of the brains (see Table 3.8-1) showed the pineal had not been removed in one pinealectomy treated tammar (No. 560) so it was excluded. The pineal was present in all the sham operated animals and damage to the pineal was only seen in one (No. 4950).

(iii) Results

Birth/oestrus after surgery

Pinealectomy in October failed to abolish seasonal quiescence as birth/oestrus in all tammars occurred after December 22. (Figure 6.1-1; Table 6.1-2). Seasonal quiescence was also retained in all of the sham operated animals. However, the interval to birth/oestrus after pinealectomy was significantly less than after sham pinealectomy (Table 6.1-2).

Progesterone profiles

In all animals, progesterone was elevated prior to birth/oestrus (Figures 6.1-3 to 6.1-6; Appendix B.5) as reported for intact animals (Hinds and Tyndale-Biscoe, 1982a).

Pouch condition

Pouch cleaning, and increased pouch moisture usually precede birth/oestrus and was detected in most animals (Table 6.1-2).

This was detected in Sham PINX No. 510 from January 21, but birth/oestrus was not detected. However, as a precipitous decline in plasma progesterone was seen from January 20 - 27 (Figure 6.1-5), and such a decline precedes birth/oestrus, this animal was considered to have undergone a pregnant or oestrous cycle. Oestrus was therefore assumed to have occurred on January 26, 28 days before birth on February 24 (Table 6.1-2).

Prolactin profiles

The plasma prolactin concentrations measured at weekly intervals remained constant in both the pinealectomy and sham pinealectomy groups until February 26 when they decreased to less than 20ng/ml (Figure 6.1-7; Appendix B.1). No significant differences between the groups were recorded.

The circadian profile of plasma prolactin measured both pre- and post-operatively also showed no significant changes within each treatment throughout a 24 hour period (Figure 6.1-8 and Appendix B.2). However, an analysis of variance of these profiles indicated a significant difference between the three treatments (Appendix B.3). Using the Newman-Keuls statistical test for *a posteriori* comparisons, (Mendenhall and Ramey, 1978, p.244), significant differences were found between the sham pinealectomy and pre-operative profiles, and the sham pinealectomy and pinealectomy profiles only. The profile of the pinealectomy group was not significantly different to the pre-operative profile (Appendix B.4).

Reproductive status at autopsy

Pinealectomy group

At autopsy all of the pinealectomized tammars had a pouch young, and a blastocyst was recovered from the uterus of each animal (Table 6.1-9). The weights of the associated corpora lutea ranged from 7mg to 10mg. No developing follicles were found in these animals and the two uterine weights in each animal were similar (weight ratios ~1:1).

Sham pinealectomy group

At autopsy three tammars were carrying pouch young (Table 6.1-9). Two others that did not have pouch young were in advanced pregnancy (uterine ratios 6.95:1 and 8.41:1), each having a large associated corpus luteum, and a developing follicle on the contralateral ovary (Nos. 392 and 4772). Two tammars had ovulated; in No. 510 an ovum was recovered, but no ovum or blastocyst was recovered from No. 4700. Their corpora lutea weights were 9mg, and the uterine weights of each animal were similar.

(iv) Discussion

The increases in plasma progesterone in both groups, that were associated with reactivation of the CL conformed to the profiles reported in intact animals (Hinds and Tyndale-Biscoe, 1982a; Tyndale-Biscoe and Hinds, 1984).

The low weights of corpora lutea in those animals carrying pouch young were similar to those reported during quiescence and early

gestation (Renfree, Green and Young, 1979). This and the low progesterone levels, recovery of quiescent blastocysts at autopsy, and lack of developing follicles, are indicative of lactational-induced suppression of the CL. The similar uterine weights in each animal also indicate this, as the pregnant uterus only enlarges when gestation resumes after reactivation (Renfree and Tyndale-Biscoe, 1973).

From these observations, both pinealectomized and sham operated tammars exhibited normal pregnant and oestrus cycles, lactational induced quiescence, and the hormonal changes associated with these.

Prolactin levels in each group showed no appreciable change when measured at weekly intervals after surgery, and were not significantly different between each group.

These findings are in contrast with a previous report that by December 22 the levels of intact animals had decreased to those typical of lactational quiescence (Tyndale-Biscoe and Hinds, 1984). However, pinealectomy did appear to have prevented an increase in the total daily concentrations of prolactin. The increase in the sham pinealectomy group after surgery was not seen in the pinealectomy group (Figure 6.1-8), but as pinealectomy failed to induce immediate reactivation of the CL, the prolactin levels may still have been sufficiently high to maintain diapause.

The particularly interesting finding from this study was that seasonal quiescence was retained after pinealectomy. This is in contrast to the findings of Renfree *et al.* 1981, as seasonal quiescence was abolished in tammars that were ganglionectomized in lactational

quiescence. This could have been due to 1) differences inherent in the two surgical procedures or, 2) the response to loss of pineal function differed in lactational and seasonal quiescence.

Differences between pinealectomy and ganglionectomy have been reported in the neuroendocrine sequelae and behaviour. In ovariectomized steroid-primed rats, ganglionectomy depressed serum prolactin levels, but pinealectomy did not (Cardinali *et al.*, 1979). A goitrogenic response to methylmercaptoimidazole was observed in ganglionectomized but not pinealectomized rats (Pisarev *et al.*, 1981). Synchronization of the circadian feeding rhythm of rats took longer in ganglionectomized, compared to pinealectomized animals (Baum, 1970).

Other studies, however, have demonstrated similarity between pinealectomy and ganglionectomy. Both treatments prevented the acceleration of oestrus in ferrets exposed to long photoperiods (Herbert, 1968, 1969), removed the inhibitory influence of blinding on the gonads of the Syrian hamster (Reiter and Hester, 1966), and rendered the ram insensitive to the photoperiod changes that influence its reproductive status (Barrell and Lapwood, 1979; Lincoln, 1979).

To determine whether pinealectomy or ganglionectomy had different effects on seasonal quiescence, or if there was a seasonal response to loss of pineal function, it was necessary to perform both treatments during both lactational and seasonal quiescence.

6.2 Comparison of pinealectomy and ganglionectomy.

(i) Introduction

To determine whether the apparent anomaly between the results of pinealectomy (Chapter 6.1) and ganglionectomy (Renfree *et al.*, 1981) was due to differences inherent in these procedures, or in the response to loss of pineal function at different times of year, I decided to compare the effects of each treatment in a parallel experiment (see Figure 6.2-1). That is, tammars were ganglionectomized in seasonal quiescence to compare with the previous results of pinealectomy at this time (Chapter 6.1); another group were pinealectomized in lactational quiescence to compare with the previous results of ganglionectomy at this time (Renfree *et al.*, 1981).

(ii) Materials and methods

Twelve female tammars that had their pouch young removed between June and September 1982, and were seasonally quiescent, were either ganglionectomized (N = 7), or sham operated (N = 5), between October 18 - 28, 1982 (Experiment 3, Figure 6.2-1). Their body weights ranged from 3.3 to 5.6kg. After recovery from surgery they were maintained in outside yards with sexually mature males and regular checks were made to detect birth/oestrus.

To validate the surgery, mid-light and mid-dark blood samples were taken 4 - 7 days before, and 130 - 140 days after surgery, to monitor the melatonin profile (see Table 4.8-2), and the animals were observed for the manifestation of ptosis after surgery (Table 4.8-4). Tyroxine hydroxylase activity was also measured in the excised tissue to confirm removal of adrenergic ganglion cells (Table 4.8-3). Ablation of the ganglia was confirmed in all animals.

Another eleven female tammars that were carrying pouch young were either pinealectomized (N = 6) or sham operated (N = 5) between May 31 - June 3, 1982 (Experiment 4, Figure 6.2-1). Their body weights ranged from 3.9 - 4.8kg. After the animals had recovered from surgery they were maintained in outside yards with sexually mature males. To confirm removal of the pineal, by abolition of the nocturnal rise in plasma melatonin, blood samples were taken 6 - 9 days before, and 55 - 58 days after surgery, every four hours for 32 hours (Figure 6.2-2; Appendix A.3 and A.4). The procedures were confirmed in all but one animal; No. 5234 (PINX) had no pre-operative rise which precluded proper evaluation of the post-operative profile.

As in the experiment of Renfree *et al.* (1981) the pouch young were removed (RPY) in seasonal quiescence (September 21), to determine whether pinealectomy had abolished seasonal quiescence. The animals were then checked for birth/oestrus each day from Day 27 - 31 after RPY, and then twice weekly.

(iii) Results

Experiment 3 (Ganglionectomy in seasonal quiescence)

Ganglionectomy in October failed to abolish seasonal quiescence in six of the seven tammars (Figure 6.2-3; Table 6.2-4). However, seasonal quiescence was abolished in three sham operated tammars, but retained in two others.

The time to birth/oestrus after ganglionectomy was not significantly different from that after pinealectomy performed at this time in Experiment 2 ($P>0.05$, Table 6.2-5; Figure 6.2-3).

Experiment 4

As in the study of Renfree *et al.* (1981), some tammars pinealectomized or sham operated in May - June lost their pouch young before September (Table 6.2-6). All the rest had their pouch young removed, and three pinealectomized tammars gave birth before December 22. In these three seasonal quiescence had been abolished, but it was retained in the other three pinealectomized tammars and all of the sham operated animals.

(iv) Discussion

Three sham ganglionectomized tammars gave birth 26 - 37 days after surgery, so blastocyst reactivation probably occurred in response to this treatment. Stress is implicated, but it is paradoxical that none of the ganglionectomized tammars, which were supposedly subjected to at least the same degree of stress, reactivated in response to surgery.

Ganglionectomy in October failed to abolish seasonal quiescence. These are similar results to pinealectomy in October (Chapter 6.1), indicating that there is no difference between ganglionectomy and pinealectomy at this time. Pinealectomy in lactational quiescence (May - June) however, did abolish seasonal quiescence in some animals, as did ganglionectomy at this time (Renfree *et al.*, 1981). Together, these results showed that there was little difference between ganglionectomy and pinealectomy in their effects on seasonal quiescence.

The findings that ganglionectomy in March - May abolished seasonal quiescence (Renfree *et al.*, 1981), but pinealectomy in October failed to do so (Chapter 6.1), could not therefore be explained by differences inherent in the two surgical procedures. The alternative hypothesis, that of a seasonal difference in response to the loss of pineal function, appeared to be a more likely explanation. When all the experimental results are compared, the number of tammars in which seasonal quiescence was retained increased from none of the animals ganglionectomized in March - May (Renfree *et al.*, 1981), to 50% (N = 3) of the animals pinealectomized in May - June, and 91.7%

(N = 11) of those pinealectomized or ganglionectomized in October. This suggested that the number of tammars entering seasonal quiescence increased by May - June, and after this, pineal mediated information had initiated seasonal quiescence in almost all animals.

The experiments to be presented in Chapter 6.3 were designed to test if it was pineal mediated information around June that initiated seasonal quiescence.

6.3 Pinealectomy in April and July.

(i) Introduction

Neither ganglionectomy or pinealectomy in seasonal quiescence (October) caused immediate blastocyst reactivation as birth/oestrus occurred around the start of the normal breeding season (Chapter 6.1 and 6.2). However, as either treatment in lactational quiescence abolished seasonal quiescence, the response to loss of pineal function was different in lactational and seasonal quiescence.

As the tammar is a highly photosensitive animal, the experience of short daylength around the winter solstice (June 22) may have been the pineal mediated cue that initiated seasonal quiescence. If so, pinealectomy in April, well before the winter solstice, should abolish seasonal quiescence in all animals, as did ganglionectomy at this time (Renfree *et al.*, 1981); whereas, tammars pinealectomized in July, after the winter solstice, should all retain seasonal quiescence. The present experiments were designed to test these predictions.

(ii) Materials and methods

April group

Nine tammars with pouch young were either pinealectomized (N = 6) or sham operated (N = 3) between April 20 - 22, 1983. Their body weights ranged from 4.0 - 5.8kg. Mid-light and mid-dark blood

samples were taken 1 - 3 days before, and 55 - 58 days after surgery, to measure plasma melatonin and so validate the surgery. These procedures were confirmed in all but one animal (PINX No. 5291) (Table 6.3-1; Figure 6.3-2).

July group

Eleven tammars with pouch young were either pinealectomized (N = 6) or sham operated (N = 5) between July 11 - 13, 1983. The body weights of these animals were in the range of 3.8 - 5.3kg. To validate the surgery mid-light and mid-dark blood samples were taken 4 - 6 days before, and 104 - 106 days after surgery, and plasma melatonin concentrations were determined. These procedures were confirmed in all animals (Table 6.3-3; Figure 6.3-4).

The pouch young of animals in both groups were removed on September 21, 1983 and regular checks were made to detect birth or oestrus.

(iii) Results

April group

The pouch young of some animals in both groups were lost after surgery and before RPY in September (Table 6.3-5).

Seasonal quiescence was abolished in two (50%) of the pinealectomized tammars, as they had a birth/oestrus before December 22 after the loss or removal of their pouch young (Table 6.3-5; Figure 6.3-6). Two other pinealectomized tammars retained seasonal quiescence, as they gave birth in January and February.

Seasonal quiescence was retained in all of the sham operated tammars, as two gave birth at the normal time in January, and one had not given birth by March (Table 6.3-5; Figure 6.3-6).

July group

Seasonal quiescence was abolished in three (50%) of the tammars pinealectomized in July. (Figure 6.3-6; Table 6.3-7). The other three pinealectomized tammars, and all of the sham operated animals, did not have a birth/oestrus before December 22, so had all retained seasonal quiescence.

(iv) Discussion

These results do not support the hypothesis that pineal mediated information at the winter solstice is necessary to initiate seasonal quiescence. For the hypothesis to have been supported, seasonal quiescence should have been abolished in all of the tammars pinealectomized in April, before the winter solstice, but retained in all of the animals pinealectomized in July, after the winter solstice. However seasonal quiescence was only abolished in 60% of the tammars

pinealectomized in April, but also in 50% of the animals pinealectomized in July. This meant that the seasonal quiescence phase was initiated in some animals before April, and would have been after mid-July in others, so detection of the winter solstice was not necessary for these animals.

The finding that seasonal quiescence was only abolished in 50% of animals pinealectomized in April is difficult to reconcile with the findings of Renfree *et al.* (1981), in which it was abolished in all animals ganglionectomized at this time. This indeed may reflect a difference between these treatments. Alternatively, the time of RPY in seasonal quiescence may be important. In the study of Renfree *et al.* the pouch young were lost or removed in July-August, compared with RPY in September in this study.

A difference between the effect of pinealectomy in April or July is apparent however. In the tammars in which seasonal quiescence was abolished, the interval to birth after RPY was 28-42 days for those pinealectomized in April, compared with 66-83 days for those pinealectomized in July. (Tables 6.3-5, 6.3-7; Figure 6.3-6). The delay in the July group may indicate a residual effect of the experience of short daylengths before pinealectomy. The animals pinealectomized in April would not have experienced these short photoperiods.

However, as pineal mediated information around the winter solstice was not necessary for the manifestation of seasonal quiescence in other animals, it was possible that, in these animals, seasonal quiescence was initiated by either:

1) a non-pineal mediated environmental cue , or

2) an endogenous cue.

The following study explored the first of these possibilities.

6.4 Pinealectomy and the response to a stimulatory photoregimen.

(i) Introduction

Since almost all of the tammars that were pinealectomized or ganglionectomized in October, and about half of those pinealectomized between April and July, retained seasonal quiescence, they may still have been able to detect photoperiod changes. Alternatively endogenous factors could have been operative.

To test whether pinealectomized tammars could still respond to photoperiod changes, another group of animals were pinealectomized and then exposed to a photoregimen known to induce CL reactivation in intact animals. If pinealectomized tammars were still photosensitive, birth/oestrus would have occurred in these animals about 29 - 36 days after a photoperiod change from 15L:9D to 12L:12D (see Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983).

(ii) Materials and methods

Twelve female tammars without pouch young and in seasonal quiescence, were transferred to the photoperiod pens previously described in Chapter 2.1-(ii)(a) on August 18, 1982, and maintained with four sexually mature males. The photoperiod was set at 10L:14D (Table 6.4-1).

The tammars were either pinealectomized (N = 6) or sham operated (N = 6) between September 1 - 3, 1982. To validate the surgery, mid-light and mid-dark blood samples were taken to measure plasma melatonin, 12 - 14 days before and 14 - 16 days after surgery. The procedures were confirmed in most animals (Table 6.4-2; Figure 6.4-3), but the absence of a pre-operative nocturnal rise precluded proper evaluation of the post-operative profile in three animals (Nos. 5279, 5278 and 5282).

On September 10 the photoperiod was set at 11.30'L:12.30'D to conform with the natural increasing photoperiod. In accord with the previous studies (Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983), the photoperiod was set at 15L:9D on September 19, and 40 days later it was decreased to 12L:12D (Table 6.4-1; Figure 6.4-5). The tammars were maintained on 12L:12D until February 21, 1983 when there was a power failure in the pens, and they were returned to outside yards. Regular checks were made throughout the study to detect birth/oestrus.

(iii) Results

Two pinealectomized tammars gave birth 38 days after the photoperiod change from 15L:9D to 12L:12D in October (Table 6.4-4; Figure 6.4-5). However, three other pinealectomized tammars did not have a birth/oestrus until January.

In the sham operated group two tammars gave birth 18 and 27 days before the photoperiod change. Three of the remaining tammars had a birth/oestrus 34 - 47 days after the photoperiod change to 12L:12D (Table 6.4-4; Figure 6.4-5).

(iv) Discussion

The two sham operated tammars that gave birth before the photoperiod change from 15L:9D to 12L:12D, may have reactivated in response to the stress of confinement in the photoperiod pens or to surgery. Blastocyst reactivation, suspected as being a stress mediated response, was reported in another marsupial the quokka, by Yadav (1973). However, as these tammars did not give birth until 44 and 53 days after confinement, it is probable that blastocyst reactivation was in response to surgery 29 - 38 days previously. As in a previous study (Chapter 6.2) however, it was peculiar then that none of the pinealectomized animals reacted in response to surgery.

The two pinealectomized tammars that gave birth in early December probably did so in response to the photoperiod change from 15L:9D to 12L:12D in October. The interval to birth in these animals was 38 days, which is similar to that of intact animals (29 - 36 days) exposed to the same photoregimen (Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983).

As these animals could respond to a photoperiod change without a pineal, non-pineal mediated photosensitivity may explain the persistence

of seasonality in some pinealectomized tammars exposed to natural photoperiod. However, this cannot explain the birth/oestrus in January of the other pinealectomized tammars that were maintained from October on a constant photoperiod (12L:12D). This is highly suggestive of the involvement of an endogenous rhythm and this could also explain the results of the previous studies, in which some pinealectomized animals retained seasonality.

The persistence of seasonality in the absence of changing photoperiod cues has been reported previously (Sadleir and Tyndale-Biscoe, 1977). In their study, intact tammars without pouch young that were maintained on 15L:9D from the vernal equinox, gave birth at the same time as the control group in December and January. This could also be regarded as evidence for endogenous factors, but it is possible that other environmental cues were providing the seasonal information. Although temperature changes cannot be dismissed, nutritional changes can be, as in the present study, and in the study of Sadleir and Tyndale-Biscoe, the tammars were fed the same diet throughout the experiment.

There is one other piece of evidence that would support the involvement of an endogenous rhythm. In the study presented in Chapter 6.1, birth/oestrus in the pinealectomy group was significantly earlier than in the sham operated group (Table 6.1-2). The endogenous component of the pinealectomized tammars may have been 'free-running' and so slightly out of phase with the entrained control group. If so, the zeitgeber is pineal mediated and photoperiod is implicated.

Photoperiod has been shown to be the zeitgeber for the annual rhythm of antler development in Sika deer, *Cervus nippon* (Gross *et al.*, 1974).

As discussed in Chapter 1, after pinealectomy or ganglionectomy the annual breeding cycles of various mammals persist, but they are asynchronous with the normal cycles. If an endogenous rhythm was maintaining seasonality in some pinealectomized tammars, it was of interest to see if this persisted in the year after surgery. The following study examined this possibility.

6.5 The effect of pinealectomy or ganglionectomy on seasonality in the year after surgery.

(i) Introduction

The results of the previous studies strongly suggested that endogenous factors, and perhaps extra pineal photosensitivity, were involved in the control of seasonal reproduction in the tammar. More than half of the tammars that were pinealectomized or ganglionectomized gave birth at the normal time after the summer solistice, and this was regardless of the time of year of surgery. Similarly, most pinealectomized tammars failed to respond to a stimulatory photoregimen in October, but eventually gave birth in January whilst maintained on a constant photoperiod of 12L:12D.

If endogenous factors could maintain seasonality in pinealectomized or ganglionectomized tammars in the year of surgery, it became of interest to determine if such factors would persist into the year after surgery. This was done by monitoring the response of these tammars to RPY in the year after surgery, during the two halves of the year that corresponded to lactational quiescence and seasonal quiescence of intact females.

(ii) Materials and methods

Animals

The following groups of animals surviving from the previous experiments were available for this study:

Pinealectomy or sham operation in May, June, 1982 (see 6.2)

Ganglionectomy or sham operation in October, 1982 (see 6.2)

Pinealectomy or sham operation in September, 1982 (see 6.4)

A fourth group were animals that had experienced normal photoperiod in seasonal quiescence before surgery, and were pinealectomized (N = 6) or sham operated (N = 4) between December 6 - 8, 1982. Their body weights ranged from 4.6 to 5.75kg. As in the previous studies, pinealectomy was confirmed by measuring plasma melatonin in mid-light and mid-dark blood samples taken 11 - 13 days before, and 97 - 99 days after surgery (Table 6.5-1; Figure 6.5-2). All of these tammars had a birth/oestrus in January and February, 46 - 79 days after surgery, so seasonal quiescence had been retained in the first year (Table 6.5-3; Figure 6.5-4, Group 5).

The tammars in all four groups were maintained with sexually mature males in outside pens throughout the study period from February 1983 to March 1984.

Response to RPY

To determine whether the tammars were capable of reactivation, the pouch young were removed from all lactating females on March 28 (except Group 4), and again on September 21, 1983. The animals were checked for birth/oestrus in June 1983, and 28 - 34 days after RPY in September, and then at monthly intervals until March, 1984.

(iii) Results

Response to RPY in March, 1983

All of the 12 sham operated tammars gave birth 26 - 67 days after RPY in March (Figure 6.5-4; Table 6.5-5; Appendix D.1). Apart from the four (33%) that gave birth more than 30 days after RPY, most had reactivated at RPY as would occur at this time in intact females. Of the 17 pinealectomized or ganglionectomized tammars that were RPY in March, 16 gave birth 29 - 147 days later (Figure 6.5-4; Table 6.5-5; Appendix D.1). The larger proportion that gave birth more than 30 days after RPY (81%) indicates that this response to RPY was not as quick in this group as it was in the controls, or in intact females.

Response to RPY in September, 1983

Of the 15 sham operated animals, 2 had lost their pouch young in July and August, and the young were removed from the rest in September. None of this group gave birth before December 22, 1984,

but all had a birth during January - March so had retained seasonal quiescence (Figure 6.5-4; Table 6.5-5; Appendix D.1).

Eighteen pinealectomized or ganglionectomized tammars had their pouch young removed in September, or had lost their pouch young in August. Of these, 7 tammars (39%), gave birth before December 22. Eleven (61%) gave birth or came into oestrus during January - March 1984, and so had retained seasonal quiescence, as had the sham operated animals (Figure 6.5-4; Table 6.5-5; Appendix D.1).

Comparison with results of the previous year

When the results of all the experiments in Chapter 6 are combined, they show that 89% of the sham operated tammars retained seasonal quiescence in the first year, but all did so in the second year (Table 6.5-5).

However, of the pinealectomized or ganglionectomized groups, 76% retained seasonal quiescence in the first year, but only 61% did so in the second year (Table 6.5-5). Of the 18 tammars tested in both years, 16 retained seasonal quiescence in the first year, but only 11 did so in the second year (Figure 6.5-4).

(iv) Discussion

The results clearly show that the sham operated tammars had retained seasonality into the second year. As all of these showed seasonal quiescence in the second year, the abolition of seasonal quiescence in three animals during the first year (Group 2, Table 6.5-5) was probably due to stress associated with surgery.

Seasonality was also retained into the second year in more than half of the pinealectomized or ganglionectomized tammars. As in the sham operated animals, and in intact animals, they reactivated after RPY in lactational quiescence, failed to do so in seasonal quiescence, and had a birth or came into oestrus at the normal time in January and February. If an endogenous factor was responsible for maintaining seasonality in these animals, it could be regarded as a circannual rhythm, as all of the major features of the annual breeding cycle had been retained.

However 5 tammars that had retained seasonal quiescence in the first year failed to do so in the second year. This may have been due to one of several reasons:

1) The putative circannual rhythm generator was no longer operative,
2) The animals had become insensitive to the rhythm generator, or

3) The rhythm was present but the animals were out of phase with the normal cycle; i.e. the phase of the rhythm that allowed breeding to occur (as in lactational quiescence) had shifted into the normal seasonal quiescence period.

However, the present results do not allow an evaluation of these alternatives to be made.

Until the effects of all other environmental cues on seasonality of the tammar are examined, it cannot be concluded that the species possesses an endogenous circannual thythm. However the following evidence supports that conclusion.

1) Photoperiod change is a powerful stimulus for reproduction in the tammar (Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983).

2) The effects of photoperiod on seasonality in other species are known to be mediated by the pineal (see Chapter 1).

3) After pinealectomy or ganglionectomy the only environmental factor known to control reproduction in the tammar (photoperiod) is removed, but seasonality persists in some animals; endogenous factors are implicated.

In summary, the results of all of these studies have shown that the pineal is not totipotent in it's control of seasonal breeding in the tammar. This is not unusual, and has been found in other species (see Chapter 1). However, the pineal has been shown to be necessary for the manifestation of seasonality in about half of the population.

Therefore, the pineal may play a predominant role in seasonality in some individuals of this species, but an ancillary role in others. The following chapter describes a series of experiments that examined how the pineal mediated this role in the tammar.

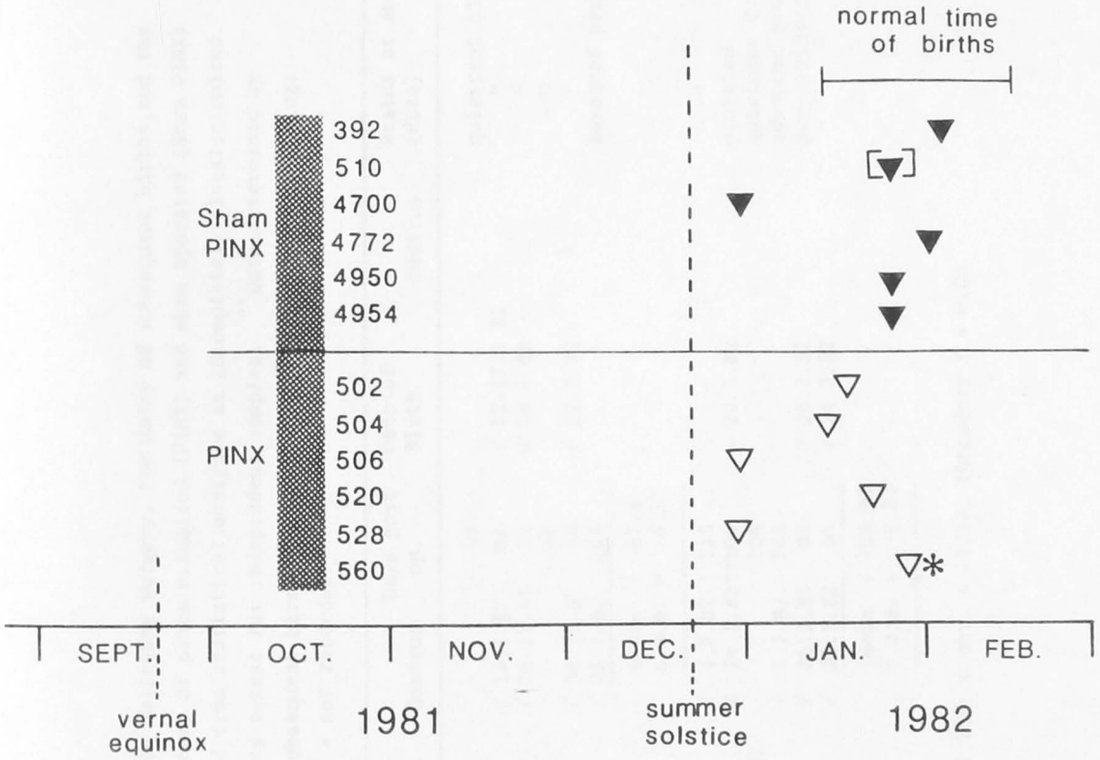


Figure 6.1-1: The times of birth/oestrus of individual tammars not carrying pouch young after PINX or Sham PINX in October, 1981. Stippled bar indicates the period of surgery. ✻No. 560 failed PINX.
Raw data and statistical analyses are given in Table 6.1-2.
▼ Birth/oestrus of Sham PINX tammar
▽ Birth/oestrus of PINX tammar
[▼] Oestrus determined from progesterone profile
All animals were sacrificed on February 25 or 26, 1982.

Table 6.1-2: The time to birth or oestrus following surgery, the dates of subsequent births, and the reproductive status at autopsy of pinealectomized (PINX) and sham operated (Sham PINX) tammars. *No. 560 excluded from statistical analysis as incompletely pinealectomized. Statistical test is students t-test for independent samples. **Date determined by progesterone profile and subsequent birth see Chapter 3.8 (iii). Pouch cleaning: ✓ = present, - = absent, ? = not recorded.

Animal Number	Treatment (date)	Date of Birth	Oestrus	Days post op.	Date of Birth	Oestrus	Status at Autopsy (date)	
502	PINX (13.10.81)	- 18.1.82		97			quiescent blastocyst	26.2.82
504	PINX (15.10.81)		✓ 15.1.82	92	✓ 12-17.2.82		" "	26.2.82
506	PINX (14.10.81)		✓ 30.12.81	77	✓ 24.1.82		" "	26.2.82
520	PINX (16.10.81)	✓ 22.1.82		98			" "	25.2.82
528	PINX (16.10.81)		✓ 30.12.81	75	✓ 27.1.82		" "	26.2.82
560	* (12.10.81)		✓ 28.1.82	108			advanced pregnancy	26.2.82
			†mean =	87.8				
			s.e.m. =	4.93				
392	Sham PINX (14.10.81)		✓ 4.2.82	113			" "	26.2.82
510	Sham PINX (13.10.81)		✓ 26.1.83**	105	- 24.2.82		ovulated	26.2.82
4700	Sham PINX (19.10.81)	✓ 31.12.82		104			quiescent C.L.	25.2.82
4772	Sham PINX (19.10.81)		✓ 1.2.82	105			advanced pregnancy	25.2.82
4950	Sham PINX (20.10.81)		✓ 26.1.82	98	? 26.2.82		pre-ovulatory	26.2.82
4954	Sham PINX (20.10.81)		✓ 26.1.82	98	✓ 24.2.82		" "	26.2.82
			†mean =	103.83				
			s.e.m. =	2.27				

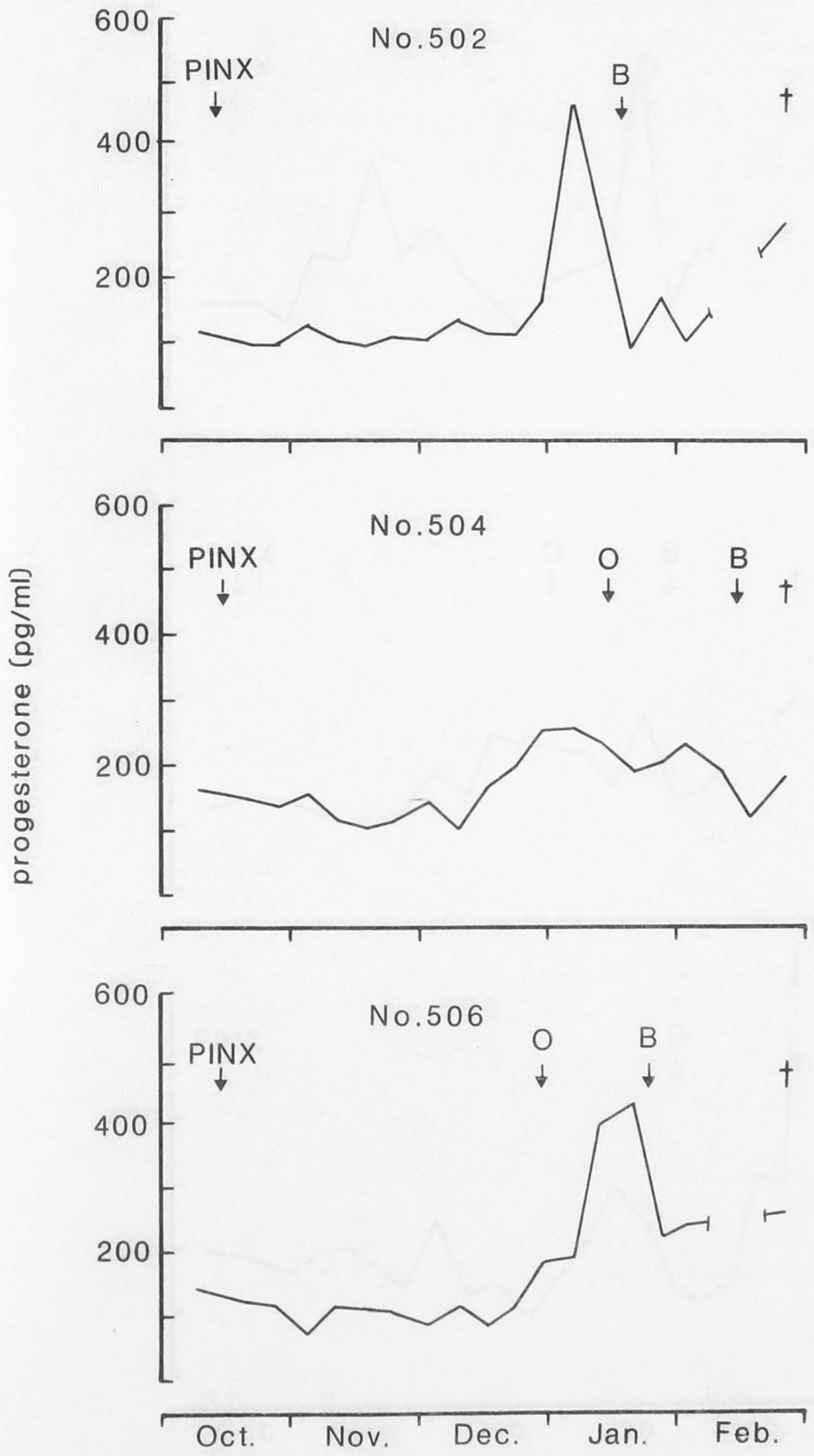
†Statistical Comparison: t 0.05, 9 = ± 2.262; t obt. = -3.14, therefore P < 0.05

Figures 6.1-3 to 6.1-6: Weekly plasma progesterone concentrations in tammars that were pinealectomized (PINX) or Sham operated (Sham PINX) in October, 1981.

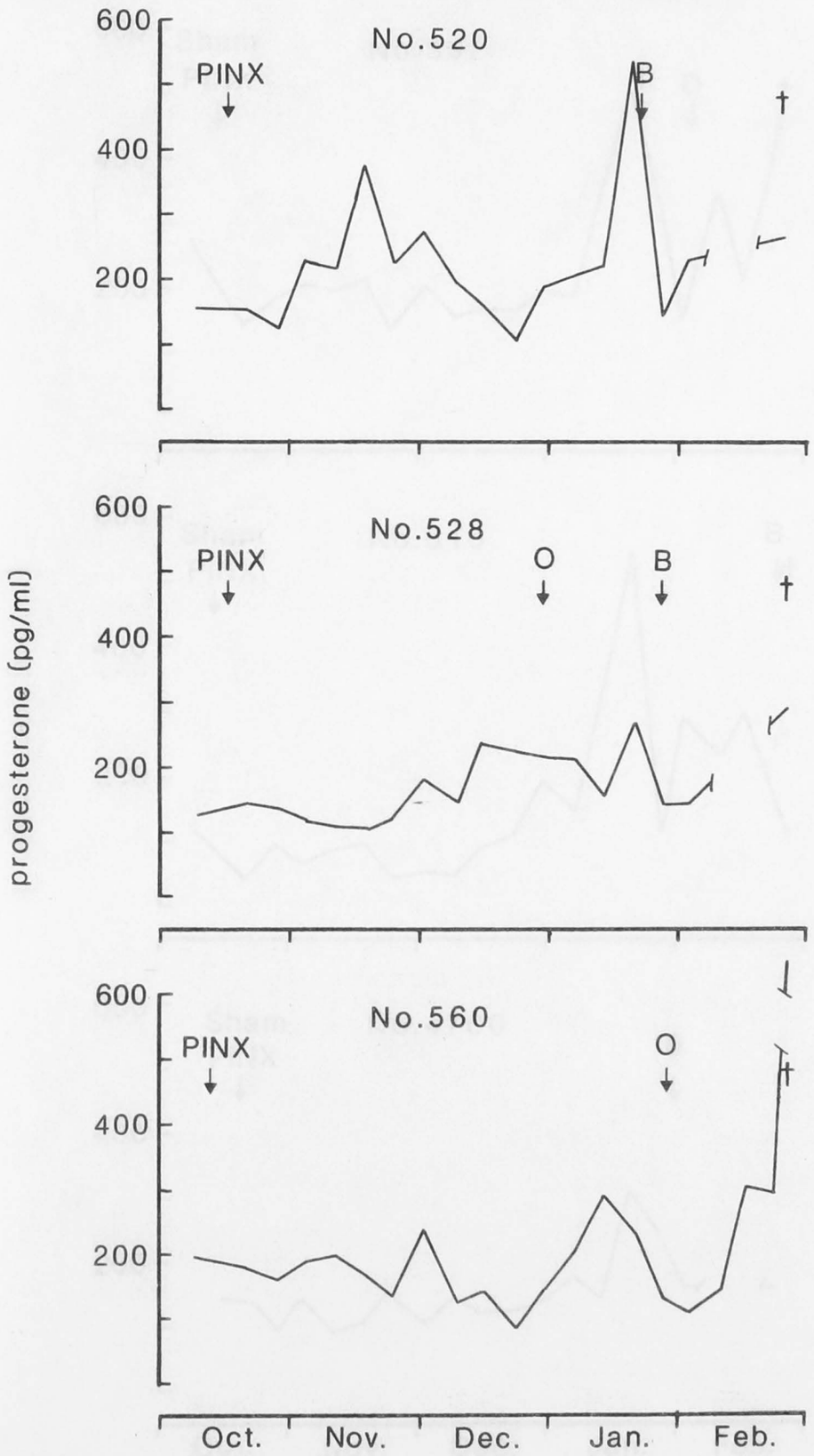
B - birth

0 - oestrus

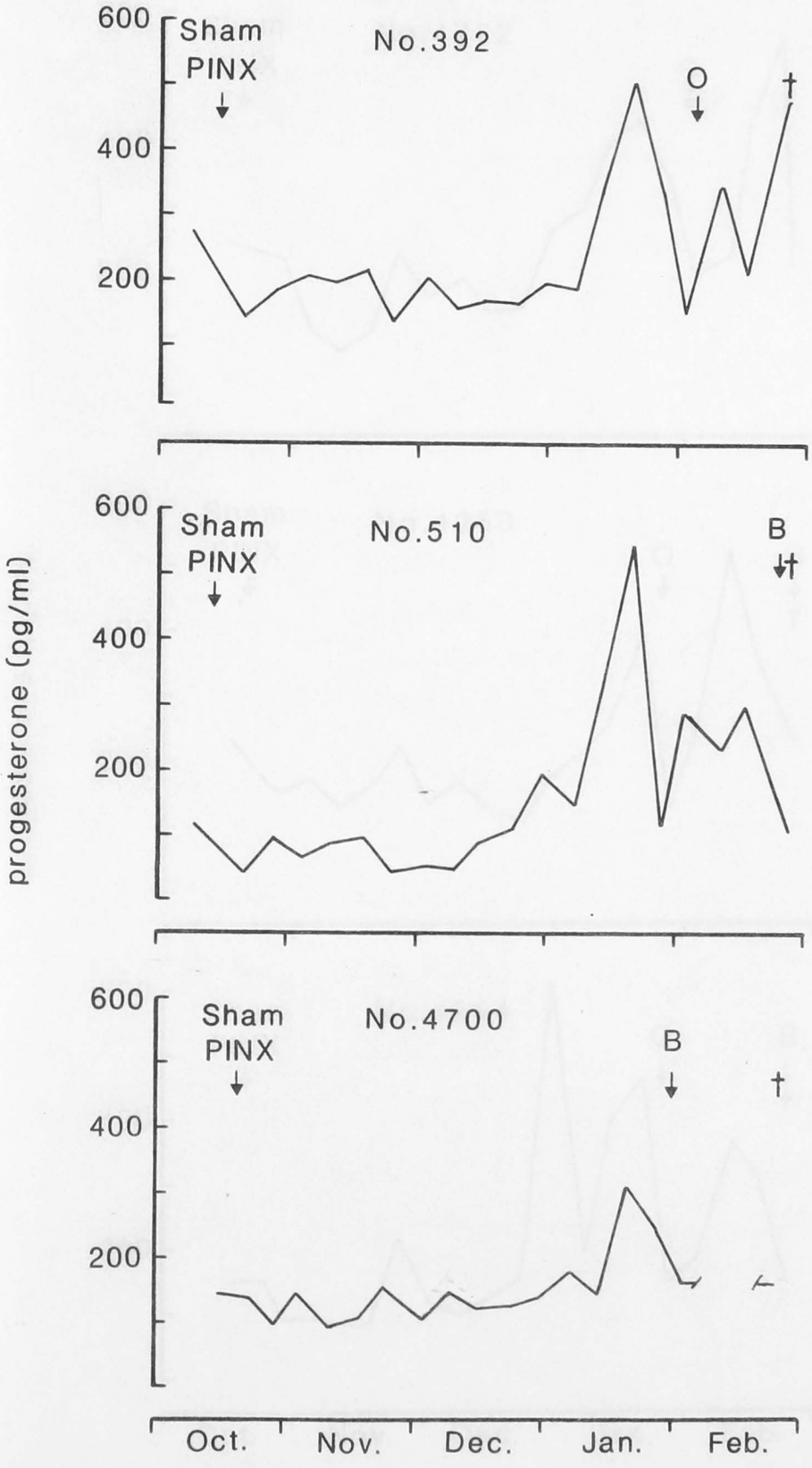
† - autopsy


600
400
200
No.502
PINX
B
†
No.504
PINX
O
B
†
No.506
PINX
O
B
†
progesterone (pg/ml)
Oct.
Nov.
Dec.
Jan.
Feb.

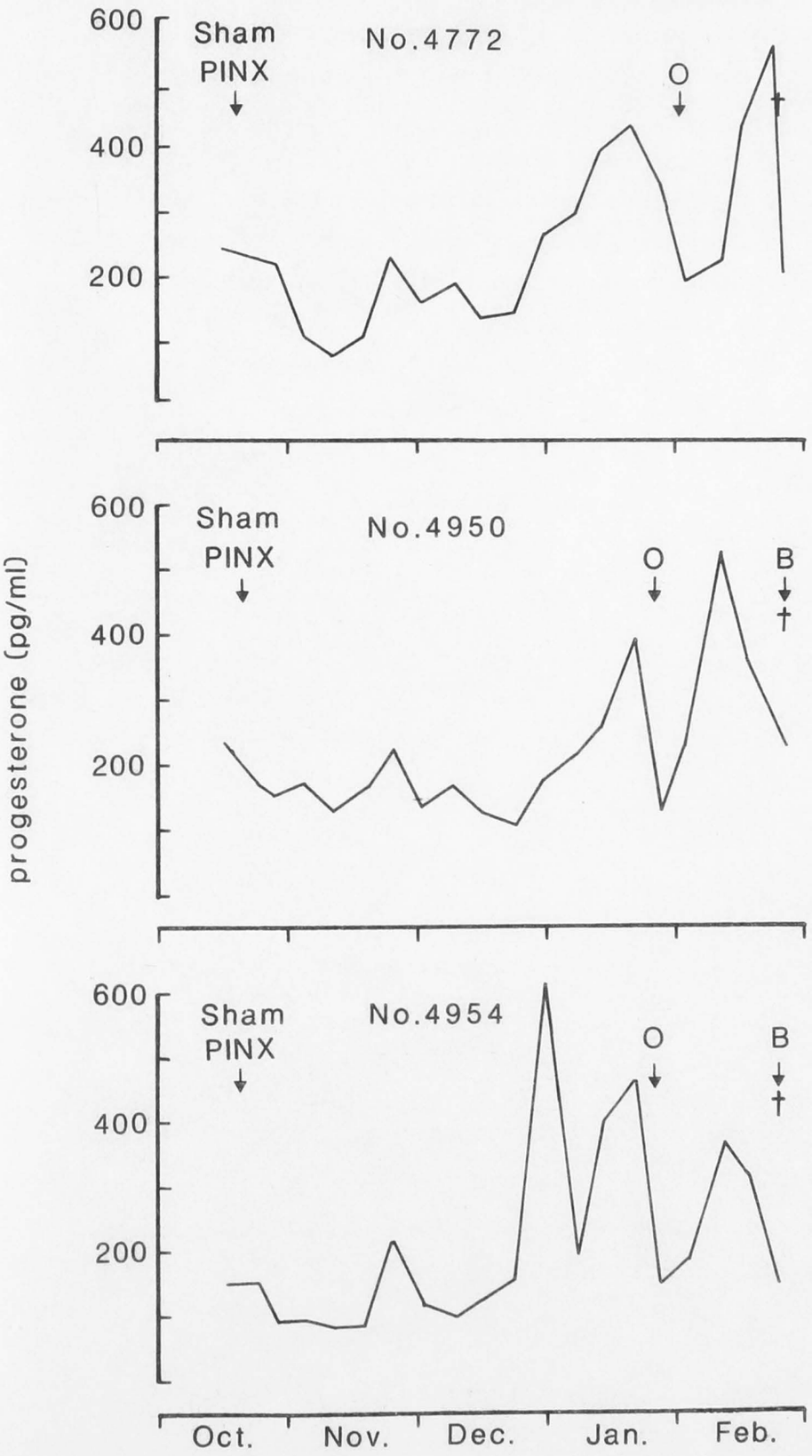
6.1-3


600
400
200
No.520
PINX
B
†
600
400
200
No.528
PINX
O
B
†
progesterone (pg/ml)
600
400
200
No.560
PINX
O
†
Oct.
Nov.
Dec.
Jan.
Feb.

6.1-4


600
400
200
Sham
PINX
No.392
O
†
600
400
200
Sham
PINX
No.510
B
†
progesterone (pg/ml)
600
400
200
Sham
PINX
No.4700
B
†
Oct.
Nov.
Dec.
Jan.
Feb.

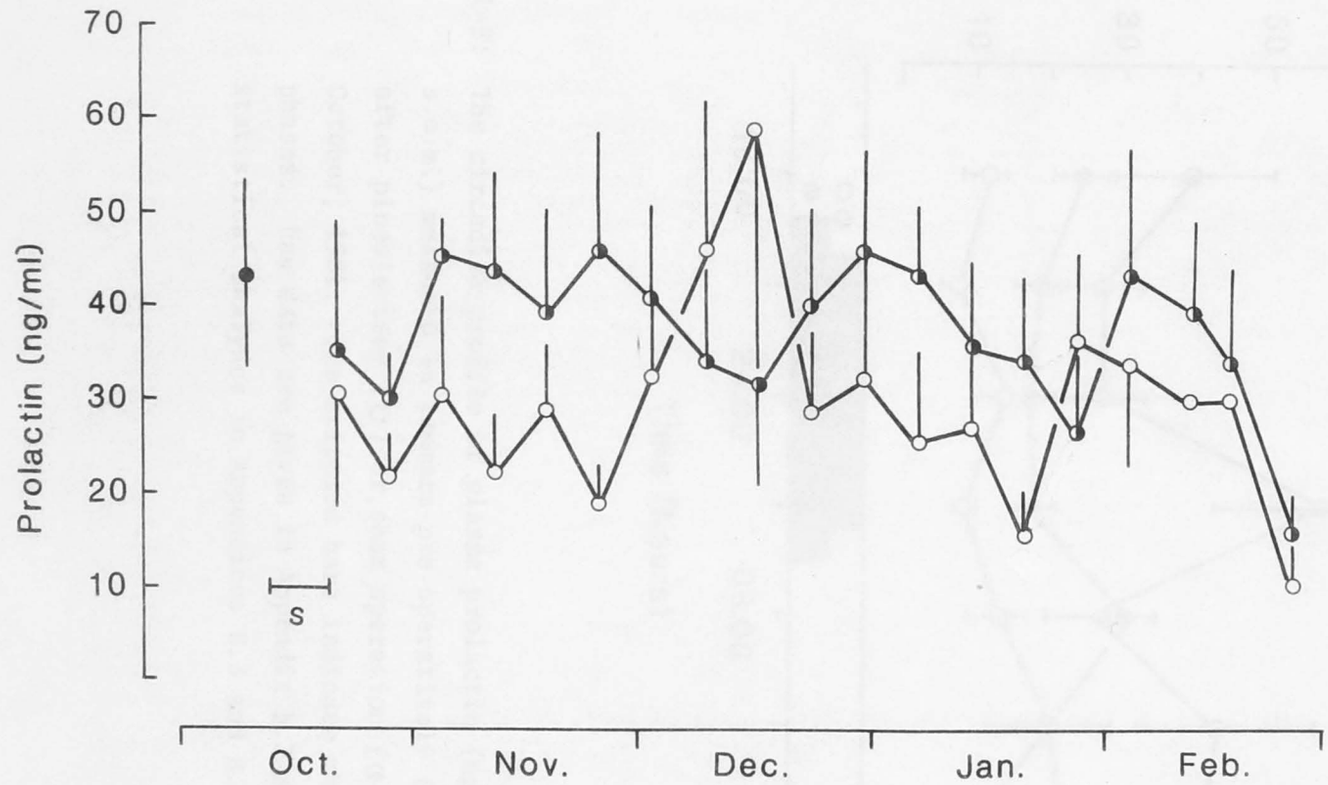
6.1-5


600
400
200
Sham
PINX
No.4772
O
†
600
400
200
Sham
PINX
No.4950
O
B
†
progesterone (pg/ml)
600
400
200
Sham
PINX
No.4954
O
B
†
Oct.
Nov.
Dec.
Jan.
Feb.

6.1-6

Figure 6.1-7: Weekly plasma prolactin concentrations in tammars measured in all animals pre-operatively (●) and after pinealectomy (○) or sham pinealectomy (◑) in October, 1981.
S - period of surgery

All time points not significantly different ($P>0.05$). Raw data and statistical analyses are given in Appendix B.1.


70
60
50
40
30
20
10
Prolactin (ng/ml)
s
Oct.
Nov.
Dec.
Jan.
Feb.

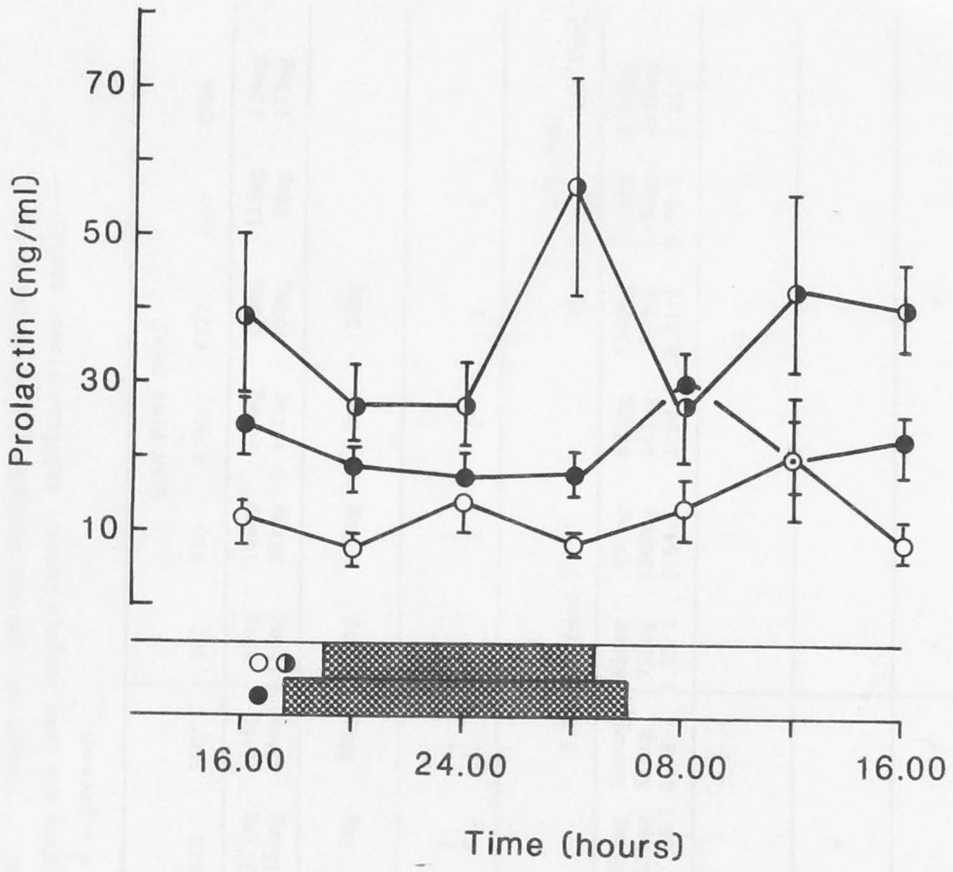


Figure 6.1-8: The circadian profile of plasma prolactin (ng/ml, mean ± s.e.m.) measured in tammars pre-operatively (●) and after pinealectomy (○) or sham operation (◑) in October, 1981. The stippled bars indicate the dark phases. Raw data are given in Appendix B.2 and statistical analyses in Appendices B.3 and B.4.

Table 6.1-9: The wet weights and sizes of various parts of the reproductive tracts of PINX and Sham PINX tammars at autopsy. *Tammar No. 560 was incompletely pinealectomized (see Chapter 3.8 (i)). Ovary weights are less corpora lutea. Follicle sizes: small, medium, large (diameter given). ✓ = present.

	PINX GROUP						SHAM PINX GROUP					
	502	504	506	520	528	560*	392	510	4700	4772	4950	4954
Rt. ovary	79mg	125mg	127mg	88mg	177mg	134mg	68mg	185mg	128mg	139mg	118mg	124mg
Lt. ovary	85mg	158mg	101mg	113mg	152mg	205mg	148mg	161mg	101mg	162mg	88mg	173mg
Corpus luteum:												
Rt. ovary	10mg			9mg	7mg	38mg	38mg	9mg	9mg	38mg		
Lt. ovary		10mg	9mg									
Corpus albicans:												
Rt. ovary		2	1	5	1	1		1				1
Lt. ovary		1		7	2	1	1	1	1	1	1	
Follicle:												
Rt. ovary											1(3.4mm)	
Lt. ovary						3.7mm	2(med.)			1(med.)		1(4.0mm)
Uterus (Rt.)	790mg	1087mg	434mg	732mg	867mg	5986mg	6636mg	877mg	685mg	7340mg	1158mg	1160mg
Uterus (Lt.)	633mg	863mg	414mg	773mg	933mg	858mg	955mg	1368mg	661mg	873mg	1480mg	1150mg
Ratio (L:R)	1.24:1	1.26:1	1.05:1	0.95:1	0.93:1	6.98:1	6.95:1	0.64:1	1.04:1	8.41:1	0.78:1	1.01:1
Ovum:												
Rt. uterus								✓				
Lt. uterus												
Blastocyst:												
Rt. uterus	✓			✓	✓							
Lt. uterus		✓	✓									
Foetus												
Rt. uterus						✓	✓			✓		
Lt. uterus												
Pouch young	✓	✓	✓	✓	✓			✓	✓		✓	

	TREATMENT	
	SCGX	PINX
Lactational quiescence	(1)	(4)*
Seasonal quiescence	(3)*	(2)

Figure 6.2-1: The design of the experiments undertaken to compare the effects of pinealectomy (PINX) or superior cervical ganglionectomy (SCGX) on seasonal quiescence.

Experiment (1) - Renfree *et al.* 1981
Experiment (2) - Chapter 6.1
Experiment (3)* - Chapter 6.2
Experiment (4)* - Chapter 6.2

*The present study.

Figure 6.2-2: The circadian profile of plasma melatonin (pg/ml, mean ± s.e.m.) measured in tammars pre-operatively (A) and after pinealectomy (B) or sham operation (C). The stippled bars indicate the periods of lights off. Raw data for each animal is given in Appendix A.3 and statistical comparisons between the light and dark samples for each group are given in Appendix A.4.

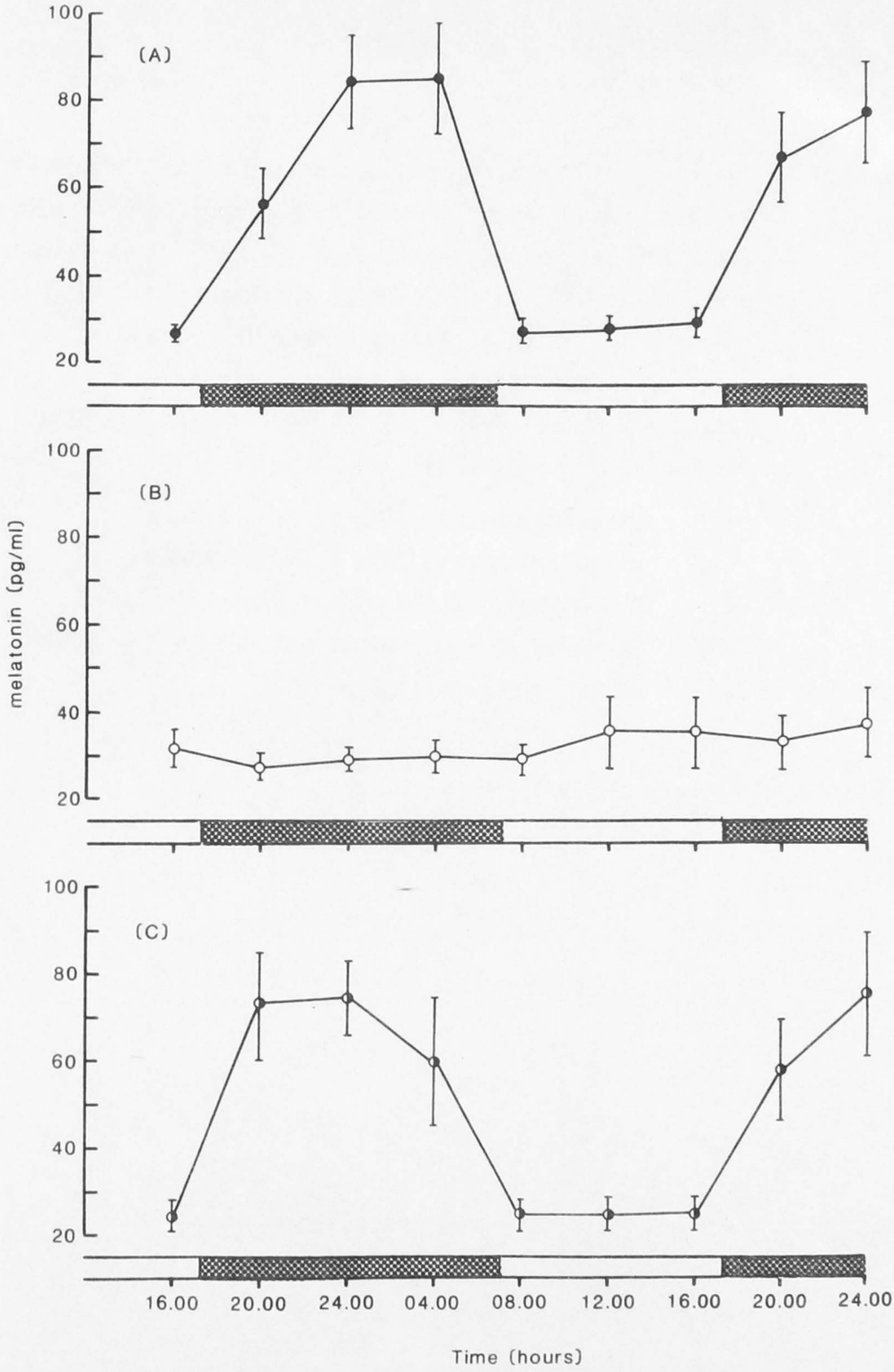

(A)
(B)
(C)
100
80
60
40
20
melatonin (pg/ml)
16.00
20.00
24.00
04.00
08.00
12.00
16.00
20.00
24.00
Time (hours)

Figure 6.2-3: The times of birth/oestrus in tammars after PINX, SCGX or sham operation in seasonal quiescence (October), and after RPY in seasonal quiescence (September) of tammars previously PINX or Sham PINX in lactational quiescence (May-June). Stippled bars indicate the period of surgery. Actual dates and analyses are given in Tables 6.1-7, 6.2-2 and 6.2-5. No. 560 (failed PINX, October 1981) is excluded from results.

▽	Birth/oestrus of PINX or SCGX tammar
▼	Birth/oestrus of Sham operated tammar
*	Pouch young of one animal lost
[▽] [▼]	Birth/oestrus of animal not RPY in September
(n)	number of animals treated.

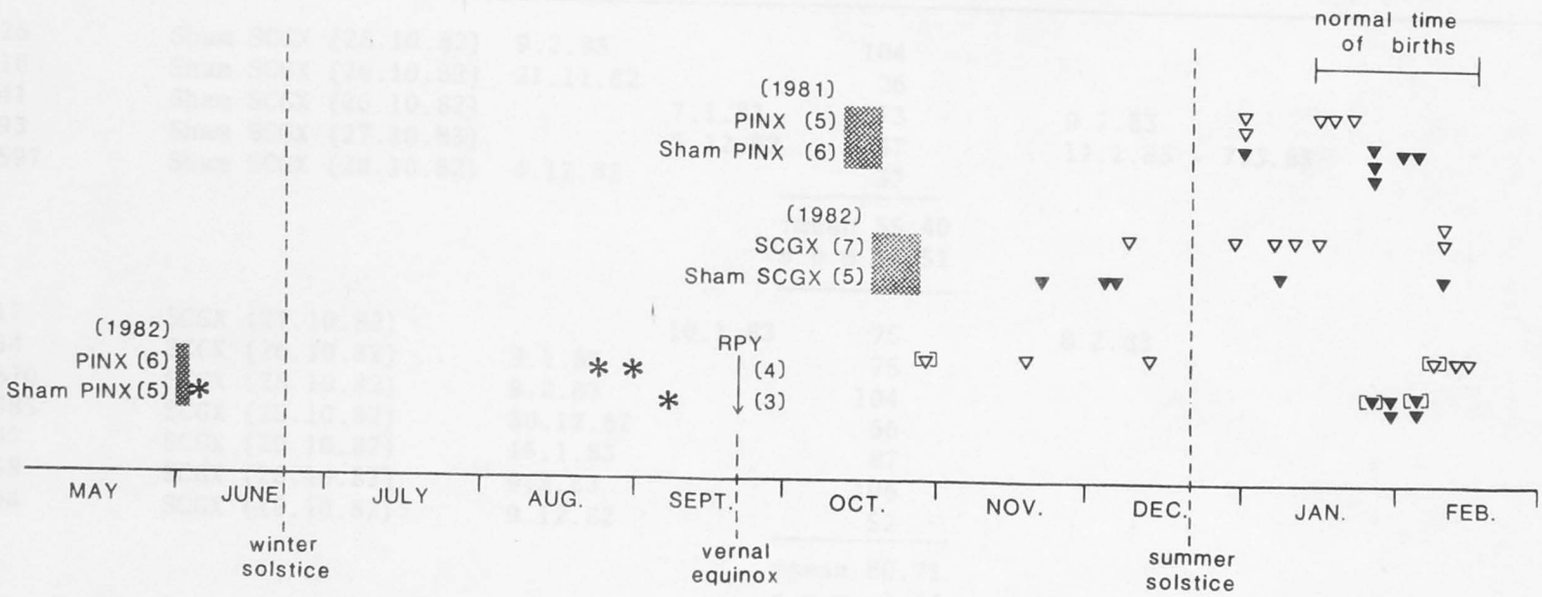

normal time
of births
(1981)
PINX (5)
Sham PINX (6)
(1982)
SCGX (7)
Sham SCGX (5)
(1982)
PINX (6)
Sham PINX (5)
RPY
(4)
(3)
MAY
JUNE
JULY
AUG.
SEPT.
OCT.
NOV.
DEC.
JAN.
FEB.
winter
solstice
vernal
equinox
summer
solstice

Table 6.2-4: The dates of birth and oestrus in tammars subjected to bilateral superior cervical ganglionectomy (SCGX) or sham operation (Sham SCGX) in October, 1982.

Animal Number	Treatment (date)	Date of Birth	Date of Oestrus	Days Post op.	Date of subsequent Birth	Date of subsequent Oestrus
526	Sham SCGX (28.10.82)	9.2.83		104		
618	Sham SCGX (26.10.82)	21.11.82		26		
641	Sham SCGX (26.10.82)		7.1.83	73	9.2.83	
693	Sham SCGX (27.10.83)		3.12.82	37	11.2.83 - 7.3.83	
5597	Sham SCGX (28.10.82)	4.12.82		37		
				†mean 55.40		
				s.e.m 14.51		
617	SCGX (27.10.82)		10.1.83	75	8.2.83	
654	SCGX (26.10.82)	9.1.83		75		
4670	SCGX (28.10.82)	9.2.83		104		
5085	SCGX (25.10.82)	30.12.82		66		
637	SCGX (20.10.82)	15.1.83		87		
658	SCGX (26.10.82)	9.2.83		106		
694	SCGX (18.10.82)	9.12.82		52		
				†mean 80.71		
				s.e.m 7.44		

†Statistical comparison: $t_{0.05, 10} = \pm 2.228$

t obt. = -1.687, therefore $P > 0.05$

Table 6.2-5: Comparison of the times to birth/oestrus after SCGX or PINX in seasonal quiescence (October). The PINX data is from Table 6.1-2.

SCGX GROUP		PINX GROUP	
Animal Number	Day of birth/oestrus after surgery	Animal Number	Day of birth/oestrus after surgery
617	75	502	97
654	75	504	92
4670	104	506	77
5085	66	520	98
637	87	528	75
658	106		
694	52		
	mean 80.71		mean 87.8
	s.e.m. 7.44		s.e.m. 4.93

$t_{0.05, 10} = \pm 2.228$

t obt. = -0.72 therefore $P > 0.05$

Table 6.2-6: The dates of birth/oestrus following loss or removal of pouch young in tammars pinealectomized (PINX) or sham operated (Sham PINX) in May/June, 1982.

Animal Number	Treatment (date)	Date of PY lost	Date of RPY	Date of Birth	Date of Oestrus	Days after PY lost or RPY	Date of Subsequent Birth/Oestrus
5063	Sham PINX (31.5.82)	4.6.82		~26.1.83		236	
5226	Sham PINX (31.5.82)		21.9.82	~30.1.83		131	
5238	Sham PINX (1.6.82)	~7.9.82		~4.2.83		136	
5316	Sham PINX (3.6.82)		21.9.82		~4.2.83	136	~3.3.83
5392	Sham PINX (2.6.82)		21.9.82	~30.1.83		131	
4835	PINX (3.6.82)	~24.8.82		~30.10.82		67	
5161	PINX (3.6.82)		21.9.82	~12.12.82		82	
5217	PINX (2.6.82)		21.9.82	~16.2.83		148	
5218	PINX (3.6.82)		21.9.82	~13.2.83		145	
5234	PINX (2.6.82)	~30.8.82		~9.2.83		163	
5336	PINX (1.6.82)		21.9.82		~19.11.82	59	17.12.82

~ estimated dates

Table 6.3-1: Pre-and post-operative mid-light and mid-dark levels of plasma melatonin (pg/ml) in tammars pinealectomized (PINX) or sham operated (Sham PINX) in April, 1983. Confirmation of surgery is made by comparison of the pre-and post-operative profiles.

	PRE-OPERATIVE				POST-OPERATIVE			
Animal Number	Light (10.00hrs)	Dark (22.00hrs)	Ratio L:D	Intended Procedure	Light (11.00hrs)	Dark (23.00hrs)	Ratio L:D	Procedure Confirmed
523	<31.3	62	1:1.98	Sham PINX	37	80	1:2.16	Yes
4988	<31.3	73	1:2.33	Sham PINX	<31.3	220	1:7.03	Yes
5005	<31.3	42	1:1.34	Sham PINX	<31.3	61	1:1.95	Yes
				mean	33.20	120.33ns	1:3.62	
				s.e.m.	1.90	50.13		
543	32	96	1:3.00	PINX	<31.3	<31.3	1:1	Yes
4853	<31.3	115	1:3.67	PINX	<31.3	<31.3	1:1	Yes
4965	<31.3	63	1:2.01	PINX	48	38	1:0.79	Yes
5057	39	175	1:4.49	PINX	78	63	1:0.81	Yes
5291	33	125	1:3.79	PINX	58	94	1:1.62	No
5370	43	97	1:2.26	PINX	53	47	1:0.89	Yes
mean	33.72	94.22*	1:2.79		48.32	42.12ns	1:0.90	
s.e.m.	1.43	13.49			8.61	5.96		

Statistical comparisons between light and dark values using students t-test for dependent samples:

All animals pre-operative: t 0.05, 8 = $\pm$ 2.306, t obt. = -4.72 therefore P < 0.05*
Sham group post-operative: t 0.05, 2 = $\pm$ 4.303, t obt. = -1.71 therefore P > 0.05 n.s.
PINX group post-operative (excluding 5291): t 0.05, 4 = $\pm$ 2.776, t obt. = 2.13 therefore P > 0.05 n.s.

Figure 6.3-2: Pre- and post-operative mid-light and mid-dark concentrations of plasma melatonin in tammars that were PINX or Sham PINX in April, 1983. Values given are the mean ± s.e.m.

Raw data and statistical analyses are given in Table 6.3-1.

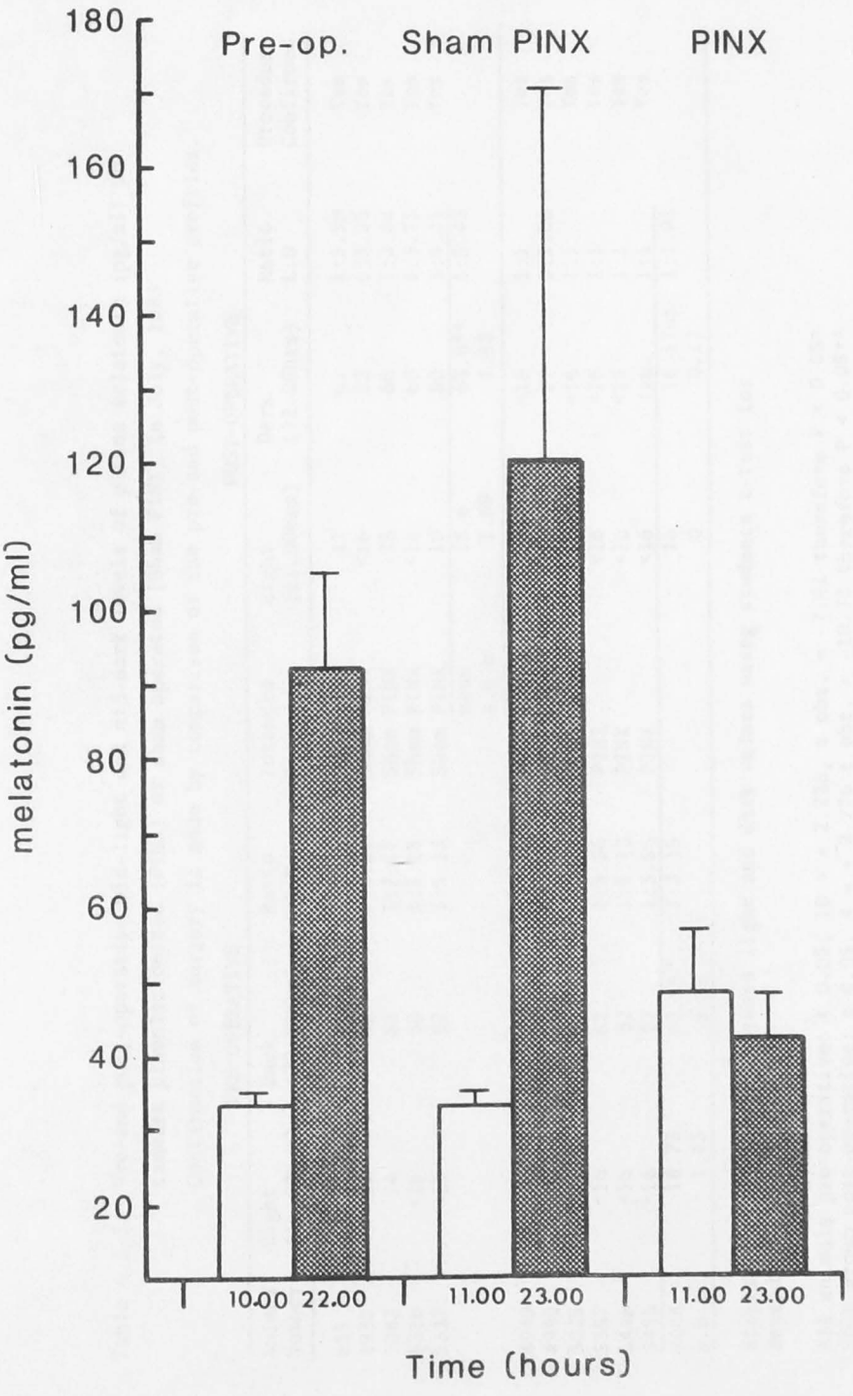

180
160
140
120
100
80
60
40
20
melatonin (pg/ml)
Pre-op.
Sham PINX
PINX
10.00 22.00
11.00 23.00
11.00 23.00
Time (hours)

Table 6.3-3: Pre-and post-operative mid-light and mid-dark levels of plasma melatonin (pg/ml) in tammars pinealectomized (PINX) or sham operated (Sham PINX) in July, 1983. Confirmation of surgery is made by comparison of the pre-and post-operative profiles.

	PRE-OPERATIVE				POST-OPERATIVE			
Animal Number	Light (11.00hrs)	Dark (22.00hrs)	Ratio L:D	Intended Procedure	Light (11.00hrs)	Dark (22.00hrs)	Ratio L:D	Procedure Confirmed
635	<16	35	1:2.19	Sham PINX	17	61	1:3.59	Yes
4938	<16	69	1:4.31	Sham PINX	<16	52	1:3.25	Yes
5083	24	52	1:2.17	Sham PINX	25	66	1:2.64	Yes
5326	<16	50	1:3.13	Sham PINX	<16	60	1:3.75	Yes
5337	<16	82	1:5.13	Sham PINX	19	80	1:4.21	Yes
					mean	18.6	63.8**	1:3.43	
					s.e.m.	1.69	4.63		
4949	28	50	1:1.86	PINX	<16	<16	1:1	Yes
4981	26	58	1:2.23	PINX	<16	17	1:1.06	Yes
5025	<16	96	1:3.50	PINX	<16	<16	1:1	Yes
5397	<16	67	1:3.56	PINX	<16	<16	1:1	Yes
5446	<16	82	1:5.13	PINX	<16	<16	1:1	Yes
5485	<16	57	1:3.56	PINX	<16	<16	1:1	Yes
mean	18.73	62.55*	1:3.15		16	16.17ns	1:1.01	
s.e.m.	1.43	5.38			0	0.17		

Statistical comparisons between light and dark values using students t-test for dependent samples:

All animals pre-operative: t 0.05, 10 = $\pm$ 2.228, t obt. = -7.41 therefore P < 0.05*
Sham group post-operative: t 0.05, 4 = $\pm$ 2.776 t obt. = -10.73 therefore P < 0.05**
PINX group post-operative: t 0.05, 5 = $\pm$ 2.571, t obt. = -1.00 therefore P > 0.05 n.s.

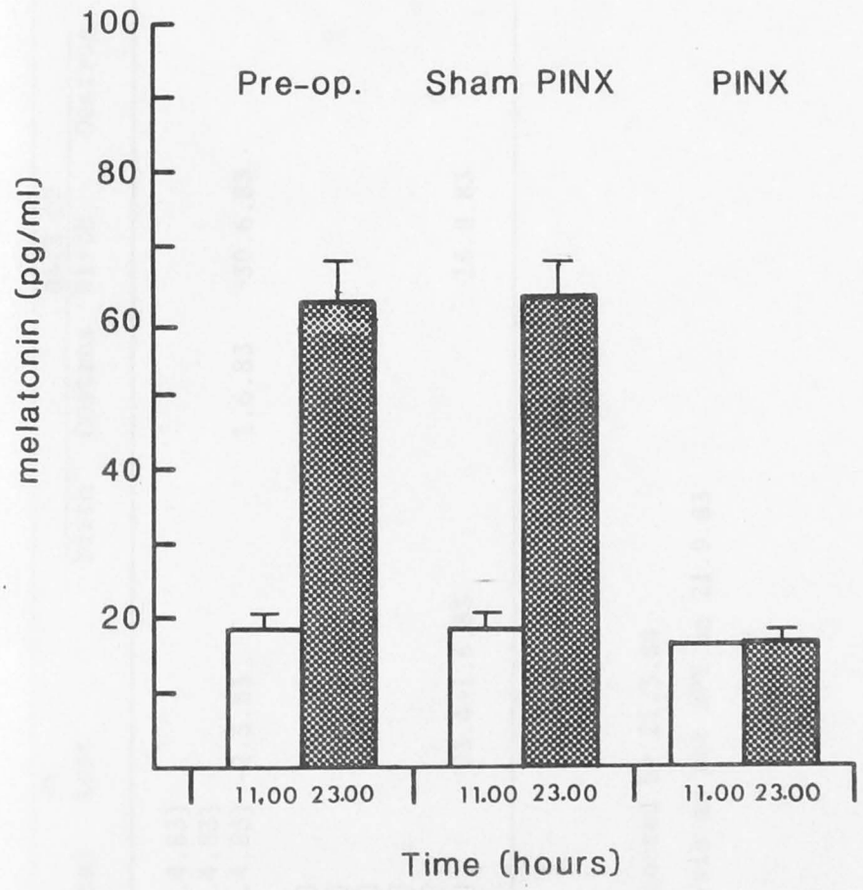


Figure 6.3-4: Pre-and post-operative mid-light and mid-dark concentrations of plasma melatonin in tammars that were PINX or Sham PINX in July, 1983. Values given are the mean ± s.e.m. Raw data and statistical analyses are given in Table 6.3-3.

Table 6.3-5: The dates of birth/oestrus in tammars PINX or Sham PINX in April, 1983.

Animal Number	Treatment (Date)	PY Lost	DATE OF				DATE OF			DAYS
			Birth	Oestrus	Birth	Oestrus	RPY	Birth	Oestrus	Post RPY
523	Sham PINX (22.4.83)						21.9.83	~29.1.84		130
4988	Sham PINX (21.4.83)						21.9.83	~7.1.84		108
5005	Sham PINX (22.4.83)	~2.5.83		1.6.83	~30.6.83		21.9.83	?		
543	PINX (21.4.83)						21.9.83	~30.1.84		131
4853	PINX (21.4.83)						21.9.83	~3.2.84		135
4965	PINX (20.4.83)	20.4.83						~9.10.83		*
5057	PINX (22.4.83)						21.9.83	~2.11.83		42
5291**	PINX (20.4.83)						21.9.83	19.10.83		28
5370	PINX (22.4.83)	23.4→1.6.83			~16.8.83		21.9.83	19.10.83		28

** failed PINX

~ estimated dates

? birth/oestrus not detected by 21.3.84

* not included in analysis as not RPY on 21.9.83

Figure 6.3-6: The times of birth/oestrus after removal of pouch young (RPY) in seasonal quiescence (September) of tammars PINX or Sham PINX in April or July. Stippled bars indicate the time of surgery. Actual dates and analyses are given in Tables 6.3-5 and 6.3-7. No. 5291 (failed PINX of April group) is excluded.

▽ - Birth/oestrus of PINX tammar

▼ - Birth/oestrus of Sham PINX tammar

[▽][▼] - Birth of animal not RPY in September

PE - Not carrying pouch young on 21.3.84

✱ - Pouch young of one animal lost

(n) - Number of animals treated

† - Animal died

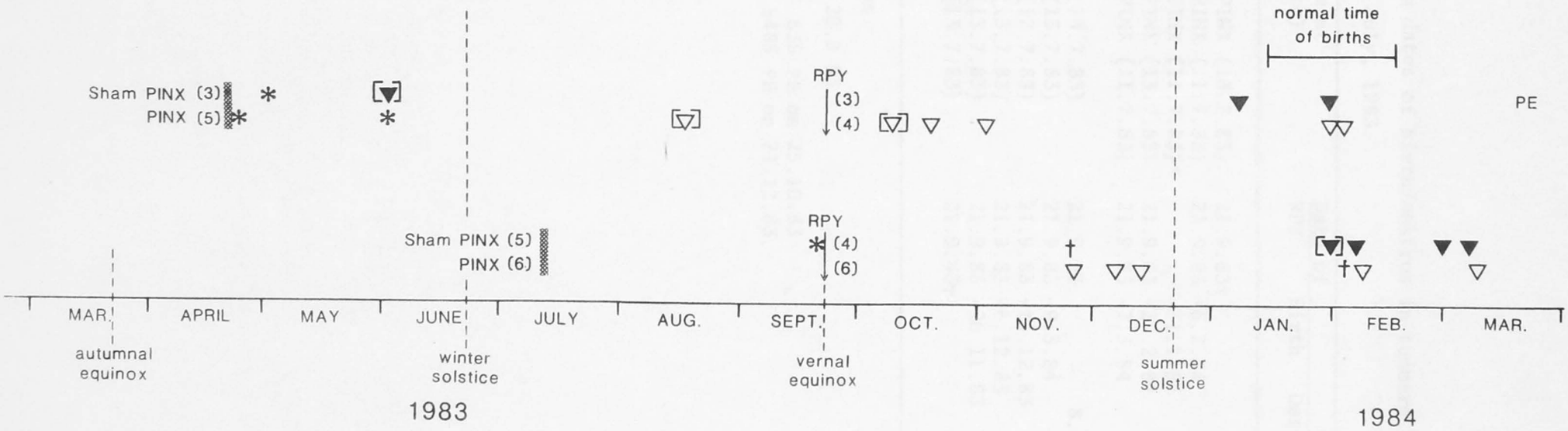

normal time
of births
Sham PINX (3)
PINX (5)
RPY
(3)
(4)
PE
Sham PINX (5)
PINX (6)
RPY
(4)
(6)
MAR.
APRIL
MAY
JUNE
JULY
AUG.
SEPT.
OCT.
NOV.
DEC.
JAN.
FEB.
MAR.
autumnal
equinox
winter
solstice
vernal
equinox
summer
solstice
1983
1984

Table 6.3-7: The dates of birth/oestrus in tammars PINX or Sham PINX in July, 1983.

Animal Number	Treatment (date)	Date of RPY	Birth	Oestrus	Days Post RPY
635	Sham PINX (13.7.83)	21.9.83†			
4938	Sham PINX (11.7.83)	21.9.83	~6.2.84		138
5083	Sham PINX (11.7.83)*		~31.1.84		*
5326	Sham PINX (13.7.83)	21.9.83	~29.2.84		161
5337	Sham PINX (11.7.83)	21.9.83	~7.3.84		168
4949	PINX (13.7.83)	21.9.83		8.2.84	140
4981	PINX (13.7.83)	21.9.83	~9.3.84		170
5025	PINX (12.7.83)	21.9.83	~13.12.83		83
5397	PINX (13.7.83)	21.9.83	~6.12.83		76
5446	PINX (13.7.83)	21.9.83	~26.11.83		66
5485	PINX (13.7.83)	21.9.83†			

~ estimated dates

* PY lost 3.8 - 20.9.83

† animal died / 635 PE on 25.10.83
5485 PE on 21.12.83.

Table 6.4-1: The photoregimen employed to test if pinealectomized tammars could respond to a photoperiod change. The tammars were pinealectomized or sham pinealectomized between Days -55 and -57.

Date (1982)	Days	Lights (E.S.T.)		Hours.mins'. of
		ON	OFF	L : D
August 18 - September 9	-71 to -49	07.00hrs	17.45hrs	10.45':13.15'
September 10 - September 18	-48 to -40	06.15hrs	17.45hrs	11.30':12.30'
September 19 - October 27	-39 to -1	06.00hrs	21.00hrs	15.0':9.0'
October 28 - February 21	0 to 116	06.00hrs	18.00hrs	12.0':12.0'

Table 6.4-2: Mid-light and mid-dark concentrations of plasma melatonin (pg/ml) in tammars measured pre-operatively and following pinealectomy (PINX) or sham operation (Sham PINX). Confirmation of surgery is made by comparison of the pre-and post-operative profiles.

	PRE-OPERATIVE			POST-OPERATIVE				
Animal Number	Light (11.00hrs)	Dark (23.00hrs)	Ratio Light:Dark	Intended Procedure	Light (11.00hrs)	Dark (23.00hrs)	Ratio Light:Dark	Procedure Confirmed
4948	<31.3	60	1:1.92	Sham	<31.3	73	1:2.33	Yes
5044	<31.3	54	1:1.73	Sham	<31.3	90	1:2.88	Yes
5279	<31.3	<31.3	1:1	Sham	<31.3	<31.3	1:1	?
5295	<31.3	60	1:1.92	Sham	<31.3	48	1:1.53	Yes
5298	<31.3	<31.3	1:1	Sham	<31.3	73	1:2.33	Yes
5401	<31.3	73	1:2.33	Sham	<31.3	100	1:3.19	Yes
				mean	31.3	69.22**	1:2.21	
				s.e.m.	0	10.48		
5278	<31.3	<31.3	1:1	PINX	<31.3	<31.3	1:1	?
5282	<31.3	<31.3	1:1	PINX	<31.3	<31.3	1:1	?
5317	<31.3	135	1:4.31	PINX	<31.3	<31.3	1:1	Yes
5347	<31.3	66	1:2.11	PINX	<31.3	<31.3	1:1	Yes
5358	<31.3	60	1:1.92	PINX	<31.3	<31.3	1:1	Yes
5427	<31.3	39	1:1.25	PINX	<31.3	<31.3	1:1	Yes
mean	31.3	56.02*	1:1.79		31.3	31.3 n.s.	1:1	
s.e.m.	0	8.44			0	0		

? = unable to evaluate surgery as no pre-operative dark rise

Statistical comparisons between light and dark values using students t-test for dependent samples:

All animals pre-operative: t 0.05, 11 = $\pm$ 2.201, t obt. = -2.93 therefore $P < 0.05$*
Sham group post-operative: t 0.05, 5 = $\pm$ 2.571, t obt. = -3.62 therefore $P < 0.05$**
PINX group post-operative: t 0.05, 5 = $\pm$ 2.571, t obt. = 0 therefore $P > 0.05$ n.s.

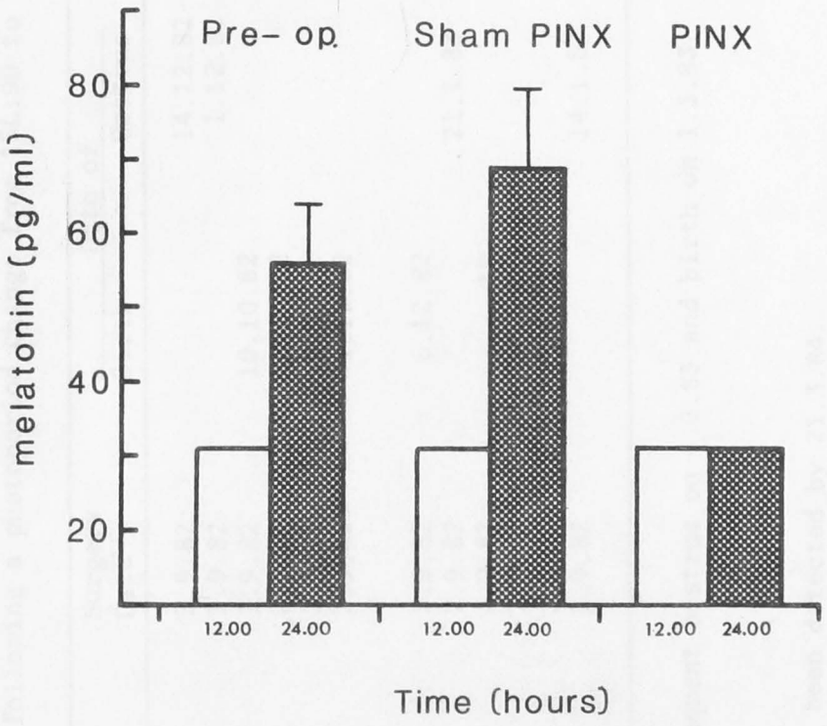


Figure 6.4-3: Pre-and post-operative mid-light and mid-dark concentrations of plasma melatonin in tammars that were PINX or Sham PINX prior to a photoperiod change from 15L:9D to 12L:12D.
Values given are the mean ± s.e.m.
Raw data and statistical analyses are given in Table 6.4-2.

Table 6.4-4: The dates of birth and oestrus in pinealectomized (PINX) and sham operated (Sham PINX) tammars following a photoperiod change from 15L:9D to 12L:12D on October 28, 1982 (Day 0).

Animal Number	Treatment	Surgery Date	Date of Birth	Date of Oestrus	Day	Date of Birth	Date of Oestrus
4948	Sham PINX	3.9.82		14.12.82	47		10.1.83*
5044	"	1.9.82		1.12.82	34 (†)		
5279	"	1.9.82	10.10.82		-18		
5295	"	2.9.82	1.10.82		-27		
5298	"	3.9.82	28.6.83		243		
5401	"	2.9.82	1.12.82		34		
5278	PINX	3.9.82	6.12.82		38 (†)		
5282	"	2.9.82		21.1.83	85	20.2.83	
5317	"	3.9.82	**				
5347	"	2.9.82	6.12.82		38 (†)		
5358	"	3.9.82	1.1.83		65		
5427	"	2.9.82		14.1.83	78	14.2.83	

*No. 4948 showed a subsequent oestrus on 4.2.83 and birth on 1.3.83.

†Denotes animal died.

** Birth/oestrus had not been detected by 21.3.84.

Figure 6.4-5: The times of birth/oestrus in tammars that were PINX or Sham PINX (◪) prior to exposure to 15L:9D then 12L:12D (---)

▽ PINX tammar birth/oestrus

▼ Sham PINX tammar birth/oestrus

Actual dates are given in Table 6.4-4

Canberra daylength (——) and the time of normal births following the summer solstice are indicated.

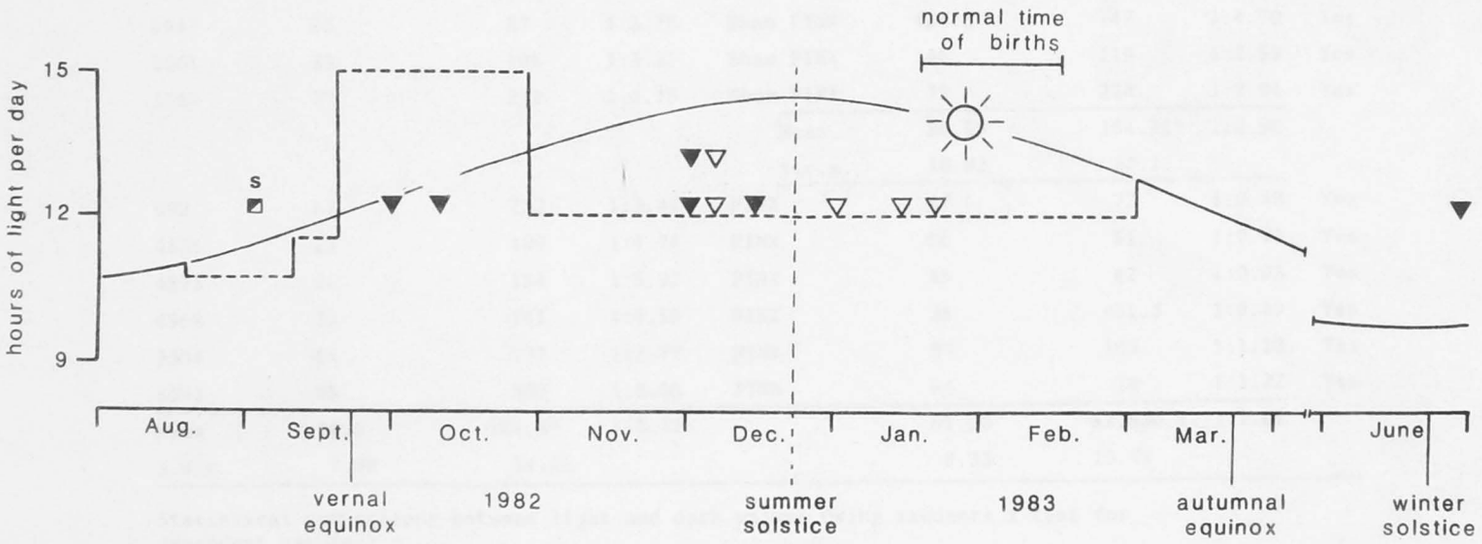

normal time
of births
hours of light per day
15
12
9
s
Aug.
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
June
vernal
equinox
1982
summer
solstice
1983
autumnal
equinox
winter
solstice

Table 6.5-1: Pre-and post-operative mid-light and mid-dark levels of plasma melatonin (pg/ml) in tammars pinealectomized (PINX) or sham operated (Sham PINX) in December, 1982. Confirmation of surgery is made by comparison of the pre-and post-operative profiles.

	PRE-OPERATIVE				POST-OPERATIVE			
Animal Number	Light (11.00hrs)	Dark (23.00hrs)	Ratio L:D	Intended Procedure	Light (11.00hrs)	Dark (23.00hrs)	Ratio L:D	Procedure confirmed
4922	92	151	1:1.64	Sham PINX	72	167	1:2.32	Yes
4947	23	87	1:3.78	Sham PINX	<31.3	147	1:4.70	Yes
5201	33	106	1:3.21	Sham PINX	46	119	1:2.59	Yes
5385	77	212	1:2.75	Sham PINX	77	224	1:2.91	Yes
				Mean	56.59	164.25**	1:2.90	
				s.e.m.	10.82	22.22		
699	62	212	1:3.42	PINX	82	72	1:0.88	Yes
4836	23	109	1:4.74	PINX	56	51	1:0.91	Yes
4873	26	154	1:5.92	PINX	45	42	1:0.93	Yes
4968	31	141	1:4.55	PINX	35	<31.3	1:0.89	Yes
5304	64	177	1:2.77	PINX	87	103	1:1.18	Yes
5341	65	199	1:3.06	PINX	64	78	1:1.22	Yes
Mean	49.6	154.8*	1:3.12		61.50	62.88n.s.	1:1.02	
s.e.m.	7.99	14.25			8.33	10.79		

Statistical comparisons between light and dark values using students t-test for dependent samples:

All animals pre-operative: t 0.05, 9 = $\pm$ 2.262 t obt. = -10.20 therefore P < 0.05*
Sham group post-operative: t 0.05, 3 = $\pm$ 3.182 t obt. = -6.84 therefore P < 0.05**
PINX group post-operative: t 0.05, 5 = $\pm$ 2.571 t obt. = -0.31 therefore P > 0.05 n.s.

Figure 6.5-2: Pre-and post-operative mid-light and mid-dark concentrations of plasma melatonin in tammars that were PINX or Sham PINX in December 1982. Values given are the mean ± s.e.m. Raw data and statistical analyses are given in Table 6.4-1.

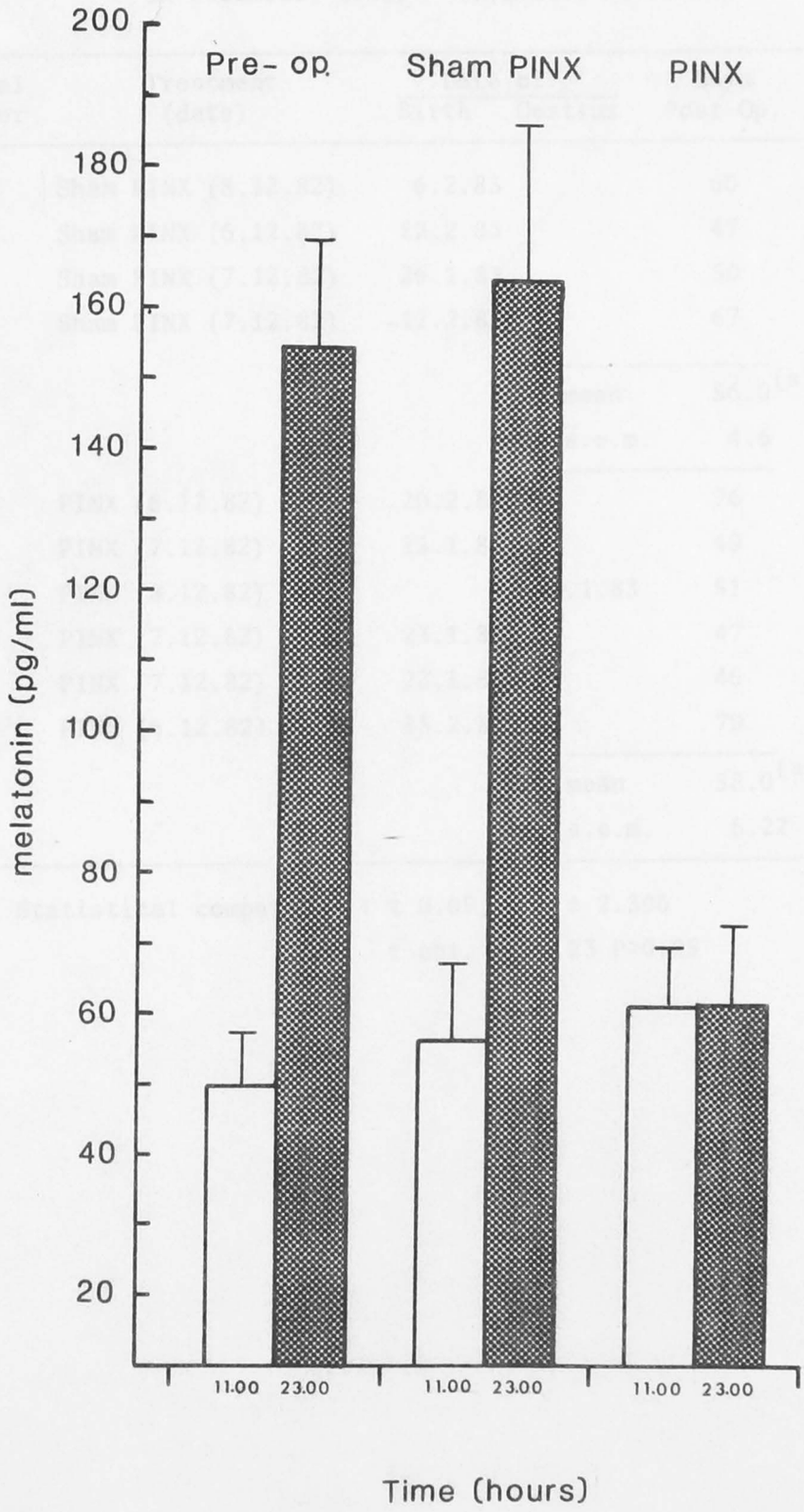

200
180
160
140
120
100
80
60
40
20
melatonin (pg/ml)
Pre- op.
Sham PINX
PINX
11.00
23.00
11.00
23.00
11.00
23.00
Time (hours)

Table 6.5-3: The dates of birth/oestrus in tammars PINX or Sham PINX in December, 1982. ~ estimated dates.

Animal Number	Treatment (date)	Date of Birth	Date of Oestrus	Days Post Op.	Date of Subsequent birth
4922	Sham PINX (8.12.82)	6.2.83		60	
4947	Sham PINX (6.12.82)	22.2.83		47	
5201	Sham PINX (7.12.82)	26.1.83		50	
5385	Sham PINX (7.12.82)	~12.2.83		67	
			mean	56.0[a]	
			s.e.m.	4.6	
699	PINX (6.12.82)	20.2.83		76	
4836	PINX (7.12.82)	25.1.83		49	
4873	PINX (8.12.82)		28.1.83	51	~27.2.83
4968	PINX (7.12.82)	23.1.83		47	
5304	PINX (7.12.82)	22.1.83		46	
5341	PINX (6.12.82)	23.2.83		79	
			mean	58.0[a]	
		-	s.e.m.	6.22	

(a) Statistical comparison : t 0.05, 8 = ± 2.306
t obt. = -0.23 P>0.05

Figure 6.5-4: The times of birth/oestrus in SCGX, PINX or sham operated tammars after surgery or RPY in seasonal quiescence in the year of, and year after surgery. Stippled bars indicate the period of surgery.

Actual dates and analyses are given in Tables 6.5-3, 6.5-5 and Appendix D.1.

Group 4 were exposed to 15L:9D,then 12L:12D from 28.10.82 to 21.2.83 (see Chapter 6.4).

▽	Birth/oestrus of PINX or SCGX tammar
▼	Birth/oestrus of Sham PINX or Sham SCGX tammar
RPY	Removal of pouch young
*	Pouch young lost
PE	Not carrying pouch young
†	Animal died
[▽] [▼]	Birth of animal not RPY in September
EXCL.	Animal excluded from analyses as died before treatment could be evaluated.

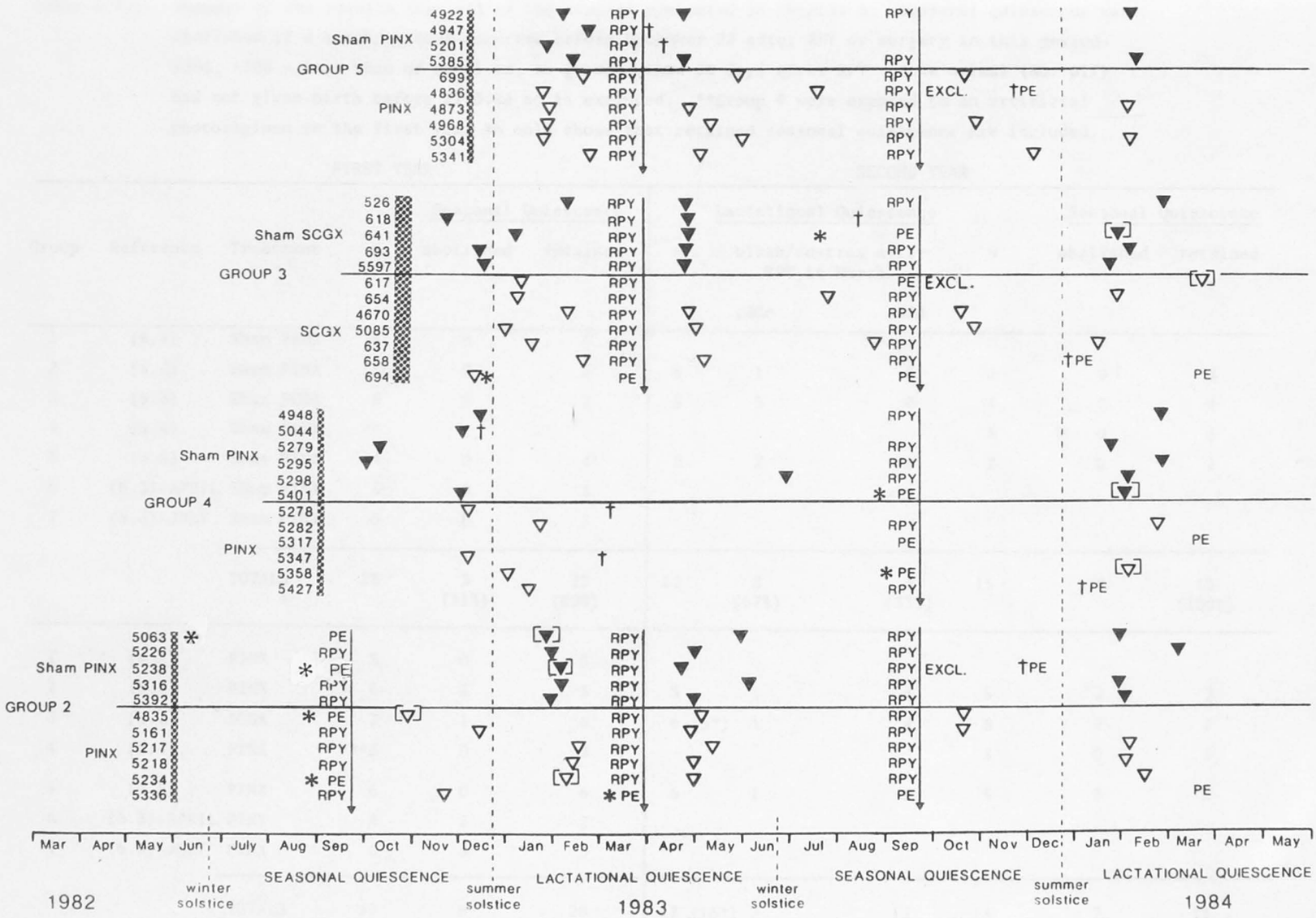

Sham PINX
GROUP 5
PINX
4922
4947
5201
5385
699
4836
4873
4968
5304
5341
Sham SCGX
GROUP 3
SCGX
526
618
641
693
5597
617
654
4670
5085
637
658
694
Sham PINX
GROUP 4
PINX
4948
5044
5279
5295
5298
5401
5278
5282
5317
5347
5358
5427
Sham PINX
GROUP 2
PINX
5063
5226
5238
5316
5392
4835
5161
5217
5218
5234
5336
RPY
PE
EXCL.
†PE
Mar Apr May Jun July Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May
SEASONAL QUIESCENCE
LACTATIONAL QUIESCENCE
SEASONAL QUIESCENCE
LACTATIONAL QUIESCENCE
winter solstice
summer solstice
winter solstice
summer solstice
1982
1983
1984

Table 6.5-5: Summary of the results from all of the studies presented in Chapter 6. Seasonal quiescence was abolished if a birth/oestrus occurred before December 22 after RPY or surgery in this period. ≤30d, >30d - less than or equal to, or greater than 30 days after RPY. *one animal (No. 617) had not given birth before 21.3.84 so is excluded. **Group 4 were exposed to an artificial photoregimen in the first year so only those that retained seasonal quiescence are included.

			FIRST YEAR			SECOND YEAR					
				Seasonal Quiescence		Lactational Quiescence				Seasonal Quiescence	
Group	Reference	Treatment	N	abolished	retained	N	birth/oestrus after RPY in March		N	abolished	retained
							≤30d	>30d			
1	(6.1)	Sham PINX	6	0	6						
2	(6.2)	Sham PINX	5	0	5	5	1	4	4	0	4
3	(6.2)	Sham SCGX	5	3	2	5	5	0	4	0	4
4	(6.4)	Sham PINX	**						5	0	5
5	(6.5)	Sham PINX	4	0	4	2	2	0	2	0	2
6	(6.3)-APRIL	Sham PINX	3	0	3						
7	(6.3)-JULY	Sham PINX	5	0	5						
		TOTALS	28	3	25	12	8	4	15	0	15
		%		(11%)	(89%)		(67%)	(33%)			(100%)
1	(6.1)	PINX	5	0	5						
2	(6.2)	PINX	6	3	3	5	1	4	5	2	3
3	(6.2)	SCGX	7	1	6	6 (5*)	1	4	5	2	3
4	(6.4)	PINX	**3	0	3				3	0	3
5	(6.5)	PINX	6	0	6	6	1	5	5	3	2
6	(6.3)-APRIL	PINX	4	2	2						
7	(6.3)-JULY	PINX	6	3	3						
		TOTALS	37	9	28	17 (16*)	3	13	18	7	11
		%		(24%)	(76%)		(19%)	(81%)		(39%)	(61%)

CHAPTER SEVEN

CIRCADIAN MELATONIN PROFILES AND THE EFFECT OF MELATONIN ADMINISTRATION

General Introduction

7.1 Seasonal changes in the circadian melatonin profile

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

7.2 Response of the melatonin profile to a stimulatory photoregimen

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

7.3 The effect of melatonin administration on seasonal quiescence

(i) Introduction

(ii) Materials and Methods

(iii) Results

(iv) Discussion

CIRCADIAN MELATONIN PROFILES AND THE EFFECT OF MELATONIN ADMINISTRATION

General Introduction

The studies presented in Chapter 6 indicated that the pineal was involved in the annual breeding cycle of the tammar, and previous studies on other species already discussed in Chapter 1 have shown that the pineal controlled seasonal breeding through changes in the circadian melatonin profile. In this Chapter I report on studies that were designed to monitor the circadian melatonin profile of the tammar at different times of the year, and in response to a stimulatory photoregimen.

Finally, melatonin was administered to test whether it could mimic the tammar's reproductive response to a stimulatory photoregimen.

7.1 Seasonal changes in the circadian melatonin profile

(i) Introduction

Seasonal changes in the circadian melatonin profile may have mediated the influence of the pineal on the annual breeding cycle of the tammar. To determine whether there were any seasonal changes, a group of tammars was exposed to a different photoperiod at four times of the year, and frequent blood samples were obtained to measure their plasma melatonin concentrations.

(ii) Materials and methods

As a decrease in daylength after summer photoperiod (15L:9D to 12L:12D), and not the summer photoperiod itself, was shown to induce blastocyst reactivation, photoperiods equivalent to those shortly after the solstices or equinoxes were used. The differences in time and daylength of these photoperiods to the preceding solstice or equinox are indicated in Table 7.1-1. Data relating to daylength was obtained from an observer's ephemerides provided by the Mt. Stromlo Observatory, Canberra, for latitude -35^{o} 19' 15.6" and longitude -9^{o}, 56' 01.4" at 0 metres. As the rate of change of daylength is greater at the equinoxes the differences in the imposed photoperiod to the environmental photoperiods are greater at the equinoxes.

Six adult female tammars were transferred to the photoperiod room described in Chapter 2.1(ii)(c) on the dates indicated in Table 7.1-1. The same six animals were used throughout the study, and their reproductive status at each time is given in Table 7.1-2.

The tammars were habituated to the imposed photoperiods for 7 days. On Day 8 blood samples were taken beginning at 16.00hrs (E.S.T.) (18.00hrs at January bleed) for two-hourly intervals until 20.00hrs on Day 9. Samples during the light were taken at 4 hourly intervals in January and April. For each sample the tammar was caught and restrained in a hessian grain bag and the blood taken via an indwelling catheter into a heparinized syringe (Chapter 2.2). On Day 10 the tammars were transferred to outside pens and kept with sexually mature males until the following sampling time. The times of sampling relative to the annual photoperiod change and breeding cycle are shown in Figure 7.1-3.

(iii) Results

The circadian profiles of plasma melatonin during each season are shown in Figure 7.1-4 and the raw data and statistical analyses are given in Appendix C.1, C.2 and C.3. Under the photoperiod of each season melatonin levels were highest during the dark phase. The onset of the nocturnal rise occurred shortly after the onset of the dark phase. A return to basal levels occurred shortly after the onset of the light phase.

An analysis of variance showed that the profile at each season was significantly different from each other profile ($P<0.01$), for all the effects and first order interactions indicated in Appendix C.2. For this test all observations are assumed to be independent, but as samples were taken repeatedly on each animal, this is not strictly correct.

However, using the standard error difference of the levels at each time point in each season, multiplied by students t (Least Significant Difference - LSD, Bliss, 1967, p.252), comparisons at each time point can be made (Appendix C.3). The nocturnal levels during January and April were generally significantly higher than at corresponding times during June and October. The nocturnal levels in October were higher than those during June. Levels during the light phase were similar at most time points in each season.

(iv) Discussion

Seasonal changes in the amplitude of the nocturnal rise of plasma melatonin were apparent. The highest levels were in summer (January) and autumn (April). Although in each season there was a marked circadian fluctuation, the levels during the dark phase remained elevated and did not fluctuate. Similarly during the light phase the levels did not fluctuate.

As discussed in Chapter 1, it now appears that it is the changes in the duration of the nocturnal rise that mediates the reproductive response to photoperiod (Kennaway *et al.*, 1982, Bittmann and Karsch, 1984). However, due to the sampling interval used in this study (two-four hourly), it was not possible to accurately determine whether seasonal changes in the duration also occurred.

Although a seasonal change in the circadian melatonin profile correlated with the annual breeding cycle, with the highest nocturnal levels occurring in the breeding season, this only suggested that

melatonin could be the pineal factor. The following studies were undertaken to test the role of melatonin in reproduction of the tammar directly.

7.2 Response of the melatonin profile to a stimulatory photoregimen

(i) Introduction

Although seasonal changes in the circadian melatonin profile were observed (Chapter 7.1), it was not possible to monitor melatonin levels during the actual period of blastocyst reactivation as this is variable at the start of the breeding season. In order to determine any changes, reactivation was induced by a photoperiod change similar to that used by Sadleir and Tyndale-Biscoe (1977), and frequent blood samples were obtained around the expected time of blastocyst reactivation. This would provide the information necessary to test whether melatonin is the hormone involved in the response of the tammar to photoperiod change.

(ii) Materials and methods

Six adult female tammars without pouch young were transferred to the photoperiod room described in Chapter 2.1-(ii)(c) on July 27, 1982. The tammars were exposed to the photoregimen detailed in Table 7.2-1. The first day of 12L:12D was designated Day 0. On Day 15 they were returned to outside pens with two sexually mature males and checked daily for births and copulatory plugs from Day 27.

Under each photoperiod blood samples were taken two hourly (Dark) and four to six hourly (Light) beginning on Days -46, -4, 0 and 5 for up

to 30 hours. Hourly samples were also taken between 18.00hrs and 22.00hrs, and again at 24.00hrs, each day for Days 0 to 5 (Table 7.2-2). Samples were also taken to measure plasma progesterone at weekly intervals from Day -36 to Day 0, daily from Day 0 to Day 5, and again on Days 9, 15 and 22. Progesterone was measured by RIA (Sernia, Hinds and Tyndale-Biscoe, 1980).

To test whether the natural increasing photoperiod experienced by these animals after transfer to outside pens prevented subsequent breeding, the pouch young resulting from the change in photoperiod were removed on November 4, 1982 (Day 46) and checks for birth or oestrus commenced 30 days later.

(iii) Results

Two tammars (Nos. 5353 and 5643) gave birth on Days -23 and -17, prior to the change from 15L:9D to 12L:12D. These are excluded from the following analyses but will be considered in the Discussion. Births were recorded for the remaining animals 31-33 days after the photoperiod change to 12L:12D (Table 7.2-3; Figure 7.2-4). The increases in plasma progesterone by around Day 20 reflected the resumption of luteal activity (Figure 7.2-5) as reported previously after a similar photoperiod change (Hinds and den Ottolander, 1983). However, the characteristic transient peak on Days 8-14 after the photoperiod change, reported by these workers, was only seen in one animal (No. 5704). The sampling interval may have been too long to detect this peak in the remaining animals. After RPY on Day 46 (November 4) birth/oestrus were not detected until January-March, 86-128 days later (Table 7.2-3).

The circadian profiles of plasma melatonin obtained under 10L:14D (Day -46), 15L:9D (Day -4) and 12L:12D (Day 0 and 5) for the first dark phase are shown in Figure 7.2-6. Raw data and statistical analyses are given in Appendices C.4 to C.7.

Under all three photoperiods melatonin was elevated during the dark phase. An analysis of variance showed that the profile under each photoperiod was significantly different from each other profile ($P < 0.001$), for all the effects and most first order interactions indicated in Appendix C.5.

The duration of the nocturnal rise decreased after the change from 10L:14D to 15L:9D, and the levels at 04.00hrs and 06.00hrs showed a significant increase (Figure 7.2-6; Appendix C.6).

After the change from 15L:9D to 12L:12D the duration of the nocturnal rise increased again. During Days 0 to 5 after this change, there was a shift in the time of onset of the nocturnal rise (Figure 7.2-6 and 7.2-7). On both Day 0 and Day 5 of 12L:12D, the nocturnal levels were significantly higher at 21.00hrs and 22.00hrs only, compared to the corresponding times under 15L:9D (Figure 7.2-6; Appendix C.6). On both Day 0 and Day 5 the onset of the nocturnal rise occurred at 21.00hrs; the nocturnal levels at each time point before this were not significantly higher than those during the light at 18.00hrs (Figure 7.2-7; Appendix C.7).

(iv) Discussion

By subtracting the normal gestation length of about 27 days, blastocyst reactivation in Nos. 5353 and 5643 would have occurred around days -50 and -44. These days were shortly after transfer from outside pens and confinement in the cages (Day -54) and the first diurnal bleed (Day -46) respectively. It is possible that stress associated with these activities induced blastocyst reactivation as has been reported in another marsupial, the quokka *Setonix brachyurus*, by Yadav (1973). Alternatively as they had experienced a decrease in daylength of 14 minutes when exposed to 10L:14D this may have been responsible.

As births in the remaining tammars were recorded on Day 32 ± 0.41 blastocyst reactivation would have occurred around Day 5 following the change from 15L:9D to 12:12D. This interval is similar to that reported previously of 29-36 days (Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983).

The failure of all tammars to reactivate in response to RPY in November, shows they had re-entered seasonal quiescence. This may have been in response to the detection of increasing daylength following their transfer to outside pens. As birth/oestrus occurred in January, February and early March, these tammars may again have been responding to decreasing daylength, but that following the summer solstice.

The sampling regime used in this study showed an increase in the duration, but not the amplitude, of the nocturnal rise of melatonin between Days 0 and 5. Of particular interest was the daily shift in the melatonin rise (Figure 7.2-7). This daily shift in melatonin secretion may reflect the 'free-running' of the suprachiasmatic nuclei which were no longer entrained by light at 22.00hrs under 15L:9D. Such 'free-running' rhythms in pineal gland function have been described for rat pineal serotonin N-acetyltransferase activity (Moore and Klein, 1974).

As blastocyst reactivation had occurred within six days after the change from 15L:9D to 12L:12D, the increase in the duration of the nocturnal melatonin rise during this period may have been the hormonal signal involved. The results of this experiment had provided the information necessary for the administration of melatonin, that was physiological in both timing and dosage, to test this hypothesis.

7.3 The effect of melatonin administration on seasonal quiescence

(i) Introduction

The results of the two previous studies suggested that either or both of the changes in the amplitude or duration of the nocturnal melatonin rise may be the signal that initiates reactivation of the CL and blastocyst. However, as an increase in the duration alone was then found to occur during the period of blastocyst reactivation, the following study examined whether this was the responsible signal. This was done by maintaining tammars on 15L:9D and administering melatonin that would mimic the increase in the duration of the endogenous rise that occurs after the change to 12L:12D.

The amplitude of the nocturnal rise of melatonin on 15L:9D was between 74 and 94mg/ml (mean values for group, Figure 7.2-6). To increase the duration of the nocturnal rise by two hours around these levels a route of administration that resulted in a rapid uptake and clearance of melatonin was needed. Ideally when administered 2 hours before dark the exogenous melatonin would be available for around 2-3 hours only, after which the endogenous melatonin rise would occur. This in effect would provide an increased duration of melatonin availability, without an increment in the amplitude. Oral administration of melatonin to sheep resulted in elevated blood levels within 30 minutes that were sustained for more than 7 hours, but subcutaneous injection in arachis oil caused a rapid rise within 15 minutes to around 300pg/ml, and clearance within 4 hours (Kennaway and Seamark, 1980). Subcutaneous administration was therefore chosen for this experiment in the tammar.

To determine the dose of melatonin that would provide a peak of approximately 100pg/ml, one twentieth of the dose used by Kennaway and Seamark (1980) was used initially, assuming the body weight of a tammar (4-5kg) was one tenth of a Merino crossbred ewe.

(ii) Materials and methods

A lactating female tammar weighing 5kg was maintained on 15L:9D (Lights ON 07.00hrs, OFF 22.00hrs) in the photoperiod room previously described in Chapter 2.1-(ii)(c).

Trial 1: Four days after confinment a catheter was inserted into the lateral tail vein and the tammar was injected subcutaneously with 5µg melatonin (1µg/kg) in 0.5ml arachis oil at 10.00hrs (Time 0). Blood samples (4.5ml) were taken at -60, 5, 10, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300 and 360 minutes. After taking off the plasma , the blood cells were resuspended in buffered sodium citrate, kept at room temperature, and returned, after each sample, at 30, 60, 150, 240 and 360 minutes as previously described (Chapter 2.2).

The injection of 5µg melatonin subcutaneously resulted in a rapid rise in peripheral plasma levels to a peak of 362pg/ml at 30 minutes (Figure 7.3-1). A return to the basal, pre-injection level occurred by 240 minutes. As the peak level was around 3.5 times the desired level the dose was decreased to 2µg melatonin.

Trial 2: The same animal was injected subcutaneously at 09.00hrs (Time 0) with 2µg melatonin (400ng/kg) in 0.2ml arachis oil subcutaneously and blood samples were taken at -5, 15, 30, 45 and 60 minutes, then at 30 minute intervals to 180 minutes, and again at 240 minutes. Resuspended blood cells were returned at each subsequent bleed.

The injection of 2µg melatonin resulted in a peak of 106pg/ml at 45 minutes, with a return to basal levels by 180 minutes (Figure 7.3-1).

Trial 3: Having determined the dose of melatonin that elevated peripheral plasma levels to around 100pg/ml, the profile obtained from injected exogenous melatonin and the endogenous nocturnal rise on 15L:9D was then established. The onset of the dark phase was advanced to 11.00hrs, to enable samples to be collected during my subjective daytime.

Thirty six days after the photoperiod phase shift, blood samples were obtained from the same tammar at 08.40, 10.00 and 11.00hrs, and then hourly to 21.00hrs. The following day 2µg melatonin in 0.2ml arachis oil was injected subcutaneously at 08.45hrs and blood samples were taken at 08.40, 09.00, 09.30, 10.00, 10.30 and 11.00hrs, and then hourly to 21.00hrs. On both days resuspended blood cells were returned at each bleed subsequent to 13.00hrs.

A nocturnal rise in plasma melatonin was observed following the phase shift of the photoperiod from lights off at 22.00hrs (Trials 1 + 2) to lights off at 11.00hrs in Trial 3 (Figure 7.3-2). The amplitude and duration of this rise was similar to that observed in animals on 15L:9D but not exposed to a phase shift (Figure 7.2-6), so it was considered a normal profile.

Subcutaneous injection of 2μg melatonin (400ng/kg) resulted in a rapid rise in peripheral levels by 15 minutes, with a peak at 45 minutes (Figure 7.3-2). A steady decline occurred over the following 2.5 hours and levels for the duration of the dark period were similar to those measured on the previous day. A return to basal levels was seen by one hour after lights on. The exogenous melatonin apparently had no effect on the normal endogenous profile, but had increased the duration of total available melatonin by 2 hours according to this sampling regime. Hence the duration of the nocturnal rise measured 5 days after a decrease in daylength from 15L:9D to 12L:12D, was mimicked by this dose of melatonin given 2.25 hours before lights off while the animals were maintained on 15L:9D (Figure 7.3-2).

Experimental design:

To test whether the observed shift in the nocturnal melatonin rise in response to decreased photoperiod caused blastocyst reactivation, twelve female tammars that were seasonally quiescent were transferred to the photoperiod pens described in Chapter 2.1-(ii)(b) on August 10, 1983 (Day -40) and exposed to 15L:9D (Lights ON 07.00hrs, OFF 22.00hrs).

The tammars were randomly assigned to two groups. One group received a subcutaneous injection of melatonin in arachis oil (400ng/kg) and the other group received the oil vehicle only. The injections were given between 19.30hrs and 19.45hrs from Day 0 to Day 14, which was 2.5 to 2.25 hours before lightsoff. On Day 15, the tammars were transferred to an outside pen with sexually mature males, and daily checks for birth or copulatory plugs began on Day 27. The times of sunrise and sunset on Day 15 were 05.38hrs and 18.09hrs (12.31'L:11.29'D) so the tammars had experienced a decrease in daylength of 2 hours 29 minutes. This protocol therefore duplicated that described in Chapter 7.2, except injections were administered two hours before dark, in place of decreased daylength to 12L:12D between Days 0 to 14.

(iii) Results

In the control group births were recorded 46 $\pm$ 1.14 days after start of injections. For the melatonin treated group this interval was 32 $\pm$ 0.84 days. Birth was not detected in one animal of each group but all animals came into oestrus (Table 7.3-3).

The interval to birth and oestrus after start of injections was significantly different between the groups ($P < 0.005$). However, the interval to birth in the control group after transfer to outside pens (Day 15) was not significantly different to the interval from start of melatonin injections ($P > 0.05$, Table 7.3-3), or after a decrease in daylength from 15L:9D to 12L:12D (c.f. Table 7.2-3; t obt. = 0.75, $P > 0.05$). The interval to birth from start of melatonin injections was also not significantly different to that after a photoperiod change from 15L:9D to 12L:12D (c.f. Table 7.2-3; t obt. = 0, $P > 0.05$; Figure 7.3-4).

(iv) Discussion

The results of this experiment indicate that melatonin is involved in mediating the decrease in photoperiod that induces blastocyst reactivation. As the melatonin injections mimicked an increase in the duration of the nocturnal rise, but not the amplitude, this may be the responsible hormonal signal. In the Syrian hamster (Tamarkin *et al.*, 1976; Trakulrungsi *et al.*, 1979), sheep (Kennaway *et al.*, 1982 ; Arendt *et al.*, 1983) and White-tailed deer (Bubenik, 1983), daily melatonin administration before dark brings about the changes in reproductive physiology and behaviour that are induced by an increase in the dark phase.

In the tammar, the melatonin treatment fully mimicked the response to a photoperiod change from 15L:9D to 12L:12D, as the interval to birth was the same as in the three previous studies (Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983; Chapter 7.2). It is interesting to note, however, that the interval to birth after photoperiod change is 6 days longer than that after RPY. From the results of Chapter 7.2, it may have been concluded that this interval was required for the onset of the nocturnal melatonin rise to reach that seen on Day 5. However, the treatment with exogenous melatonin mimicked the onset of the Day 5 profile from the first day of treatment, but the interval to birth was not decreased. The shift in the onset of the melatonin rise, resulting from either photoperiod change or the administration of exogenous melatonin, may depress plasma prolactin and so remove inhibition of the CL; prolactin concentrations have been shown to decline to basal levels within 14 days of photoperiod change, and this was the period in which the CL and blastocyst reactivated

(Hinds and den Ottolander, 1983). If melatonin made available earlier does depress prolactin, it may do so only slightly each day, and it takes 6 days for the levels to be depressed sufficiently to allow reactivation of the CL and blastocyst.

Table 7.1-1: The photoperiods used to obtain the circadian melatonin profiles of six tammars after each solstice and equinox. The times given are Australian Eastern Standard Time.

Solstice or Equinox	Date	Hours.mins. of Light:Dark	Date transferred to photopens	Difference (days)	Lights On	Lights Off	Hours.mins. of Light:Dark	Light difference (minutes) from Solstice/Equinox
winter solstice	June 22 (1982)	9.46':14.14'	June 28	+6	07.15	17.03	9.48':14.12'	+2
vernal equinox	Sept.20 (1982)	12.0':12.0'	Oct. 4	+14	05.30	18.00	12.30':11.30'	+30
summer solstice	Dec.22 (1982)	14.33':9.27'	Jan.4	+13	04.45	19.15	14.30':9.30'	-3
autumnal equinox	Mar.25 (1983)	12.0':12.0	April 7	+13	06.15	17.45	11.30':12.30'	-30

Table 7.1-2: The number of tammars carrying pouch young during exposure to seasonal photoperiods to monitor circadian melatonin profiles.

(+) - pouch young present

(0) - copulatory plug present

(-) - pouch young absent.

Animal Number	Month of blood sampling			
	June	October	January	April
639	+	-	-	+
675	+	+	-	-
679	+	+	-	0
682	+	+	-	-
684	+	-	-	-
688	+	+	-	-

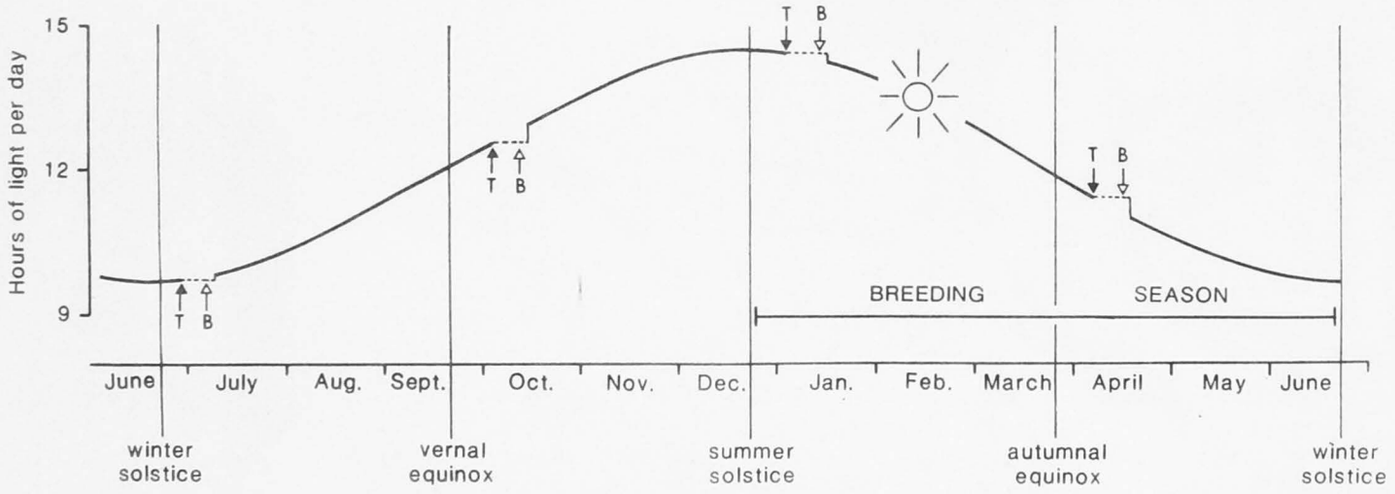


Figure 7.1-3: The times of blood sampling of tammars after the solstices or equinoxes to monitor the circadian melatonin profile during the annual breeding cycle.

—— environmental photoperiod

- - - artificial photoperiod

T- transferred to photoperiod pens

B- blood sampling

Figure 7.1-4 The circadian plasma melatonin profiles of six intact tammars exposed to winter, spring, summer and autumn photoperiods. Values given are the (mean ± s.e.m.). Raw data are given in Appendix C.1 and statistical analyses in Appendix C.2 and C.3.

	Photoperiod
□ winter (June)	9.8L:14.2D
● spring (October)	12.5L:11.5D
■ summer (January)	14.5L:9.5D
○ autumn (April)	11.5L:12.5D

Stippled bars indicate the dark phase of each photoperiod.

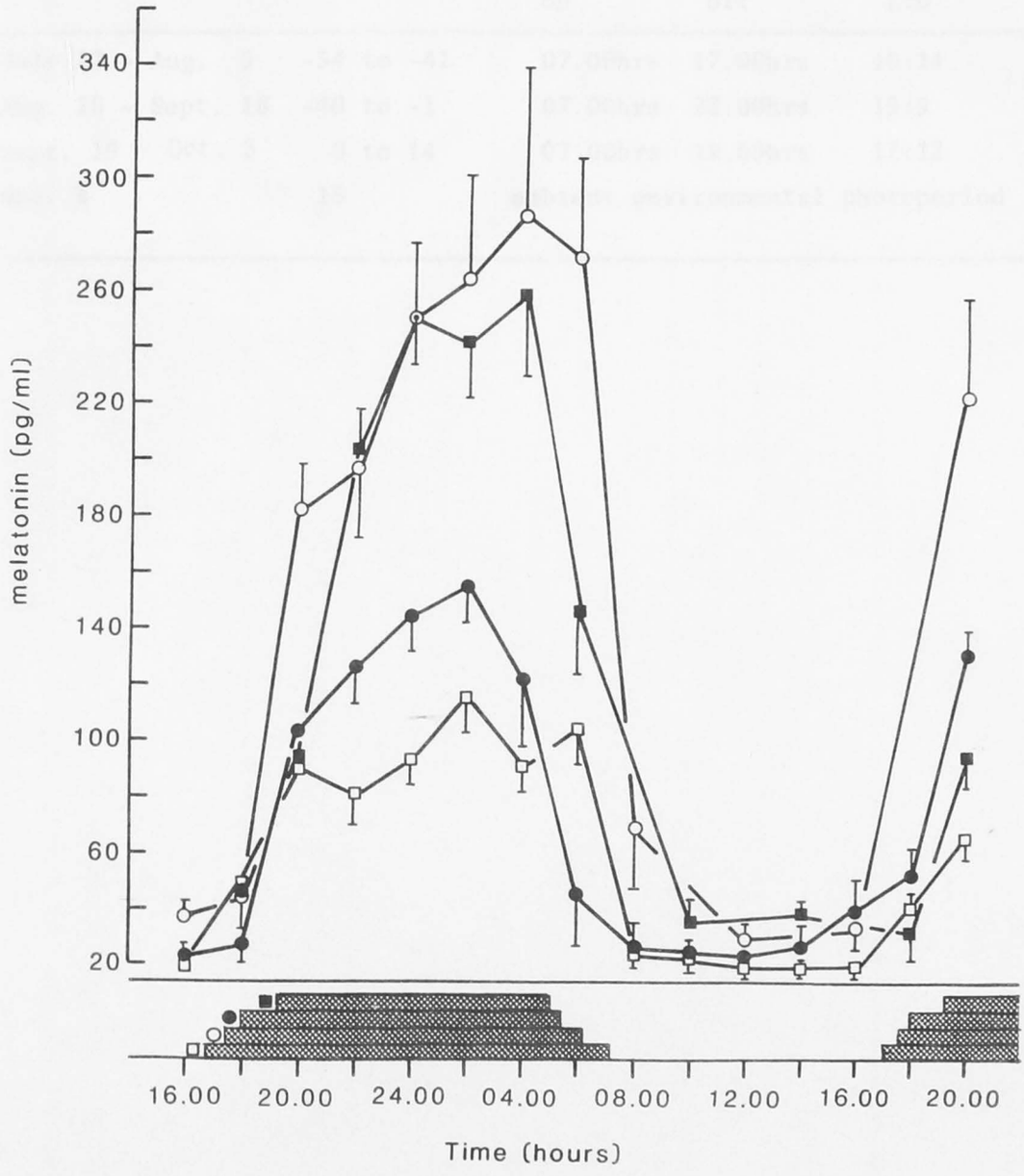

340
300
260
220
180
140
100
60
20
melatonin (pg/ml)
16.00
20.00
24.00
04.00
08.00
12.00
16.00
20.00
Time (hours)

Table 7.2-1: The photoregimen employed to monitor plasma melatonin around the time of blastocyst reactivation.

Date (1982)	Days	Lights (E.S.T.) on	off	Hours of L:D
July 27 - Aug. 9	-54 to -41	07.00hrs	17.00hrs	10:14
Aug. 10 - Sept. 18	-40 to -1	07.00hrs	22.00hrs	15:9
Sept. 19 - Oct. 3	0 to 14	07.00hrs	19.00hrs	12:12
Oct. 4	15	ambient environmental photoperiod		

Table 7.2-2: The times of blood sampling from tammars to monitor plasma melatonin (x) and progesterone (*) during exposure to a photoregimen that causes blastocyst reactivation. Encased samples taken during the dark phase.

		Time (hours)											
Day	L:D	08.00	12.00	16.00	18.00	19.00	20.00	21.00	22.00	24.00	02.00	04.00	06.00
-46	10:14			x	x *		x		x	x	x	x	x
-45	10:14	x	x	x	x		x						
-4	15:9				x *			x	x	x	x	x	x
-3	15:9	x	x		x			x	x	x			
0	12:12				x *	x	x	x	x	x	x	x	x
1	12:12	x	x		x *	x	x	x	x	x			
2	12:12				x *	x	x	x	x	x			
3	12:12				x *	x	x	x	x	x			
4	12:12				x *	x	x	x	x	x			
5	12:12				x *	x	x	x	x	x	x	x	x
6	12:12	x	x										

Table 7.2-3: The interval (days) to birth/oestrus in tammars after a decrease in daylength (15L:9D to 12L:12D) and after RPY during ambient increasing daylength.

** isolated from males, oestrus not detected

* non pregnant

( ) excluded from analysis

Animal Number	Treatment (Day 0 : Sept. 19) 1982	Day of Birth	Day of Oestrus	Treatment (Day 46 : Nov. 4) 1982	Date of Birth	Date of Oestrus	Days after RPY
5055	15L:9D to 12L:12D	32	32	RPY	2.2.83		90
5353	15L:9D to 12L:12D	(-23)	**	RPY		*15.2.83	103
5374	15L:9D to 12L:12D	33	33	RPY	12.3.83		128
5380	15L:9D to 12L:12D	32	32	RPY	29.1.83		86
5643	15L:9D to 12L:12D	(-17)	**	RPY		* 1.3.83	117
5704	15L:9D to 12L:12D	31	31	RPY	29.1.83		86
	Mean	32	32				
	s.e.m.	0.41	0.41				

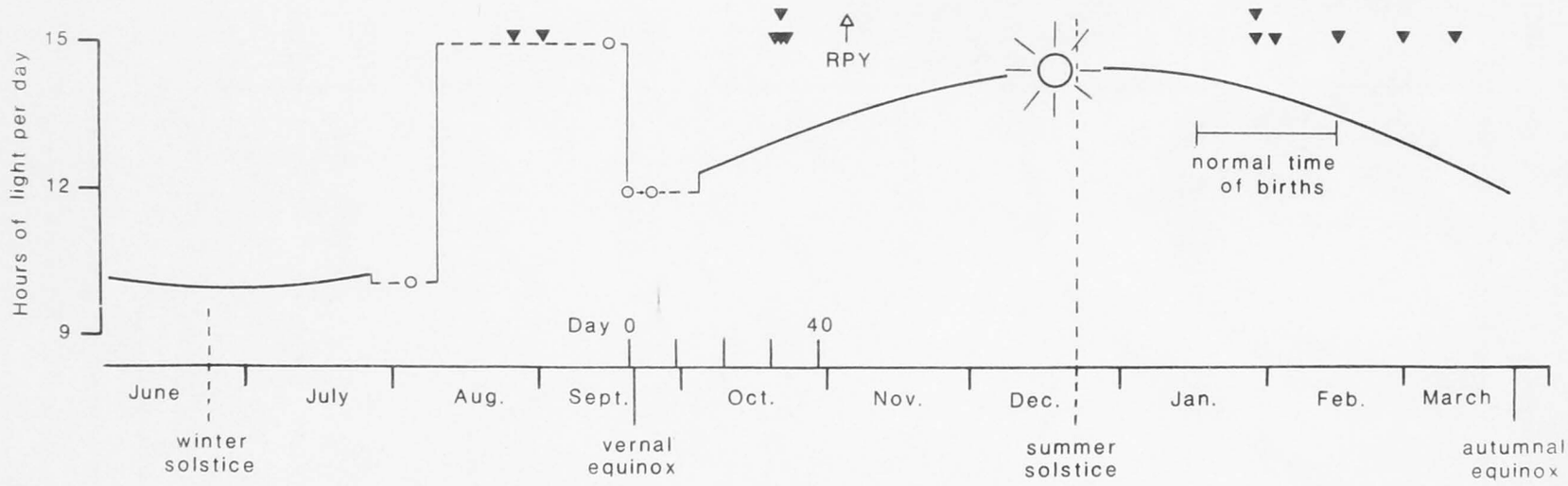


Figure 7.2-4: The photoregimen used to monitor plasma melatonin around the time of blastocyst reactivation, and the times of birth (▼) in response to decreased daylength and following removal of pouch young (RPY). Diurnal blood samples were taken each 1 - 4 hours on the days indicated by (O).

Figure 7.2-5: The plasma progesterone profiles and times of birth (▼) for 4 intact tammars exposed to a photoregime of 10L:14D, 15L:9D then 12L:12D.

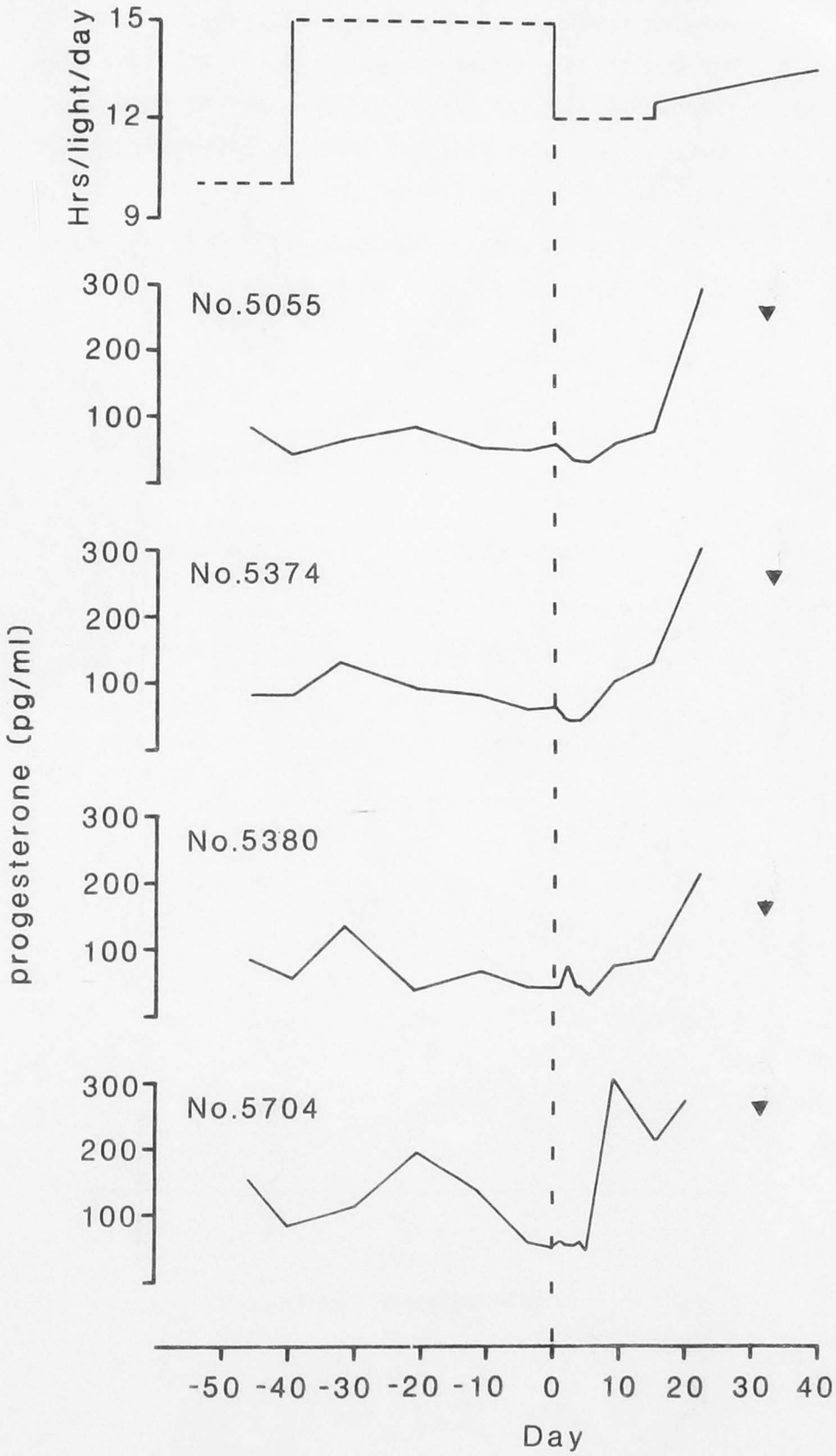

Hrs/light/day
15
12
9
progesterone (pg/ml)
300
200
100
No.5055
No.5374
No.5380
No.5704
-50 -40 -30 -20 -10 0 10 20 30 40
Day

Figure 7.2-6: Plasma melatonin concentrations (mean ± s.e.m.) for 4 tammars under a photoregime of 10L:14D (□---□), 15L:9D (■——■) and 12L:12D (●——●). Note the earlier onset of the nocturnal rise on Day 0 and Day 5 after the change from 15L:9D to 12L:12D. The stippled bars indicate the dark phase of each photoperiod. Raw data and statistical analyses are given in Appendices C.4 to C.7.

* - significantly different ($P < 0.05$) to corresponding times of 10L:14D photoperiod.

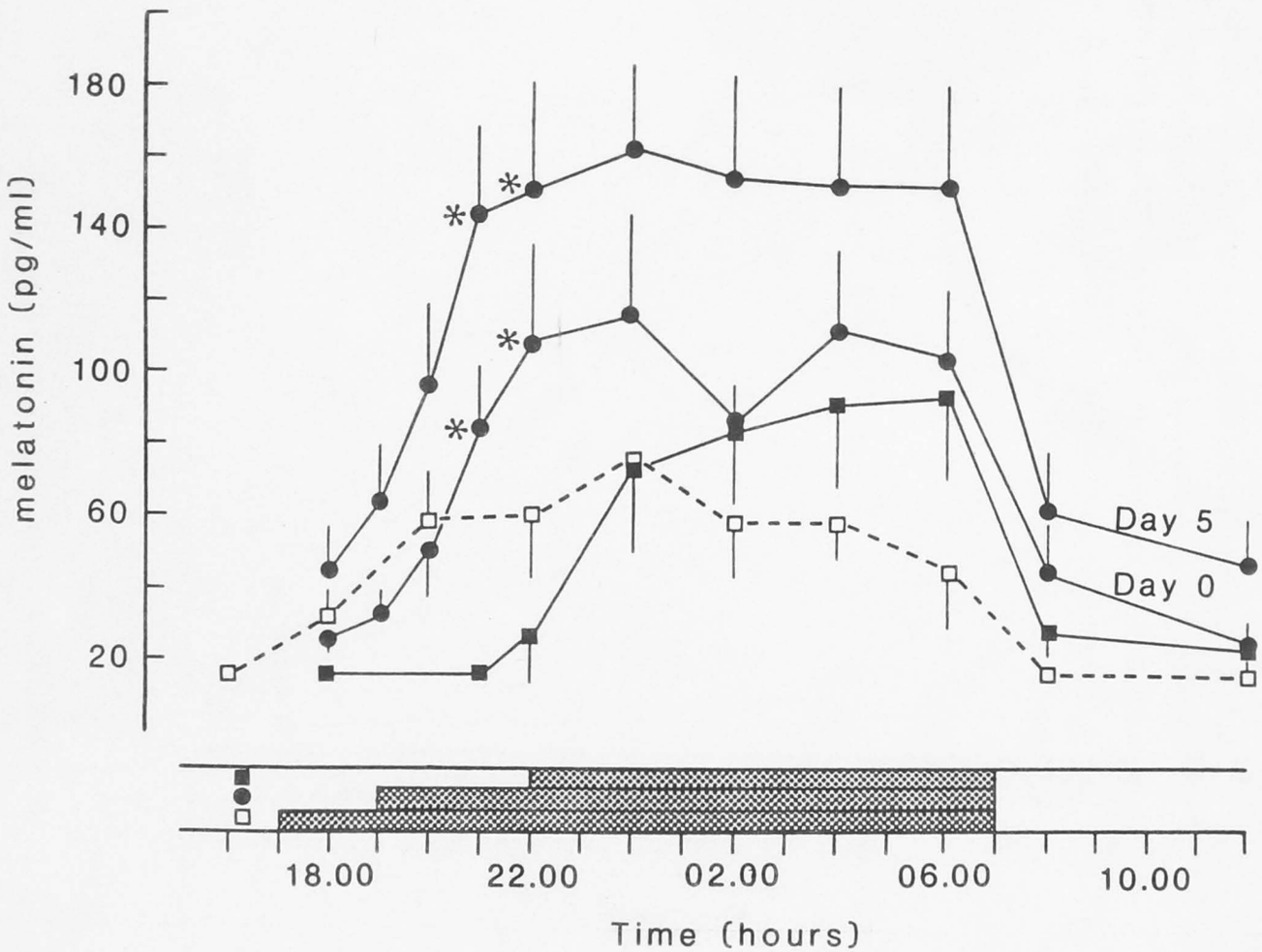
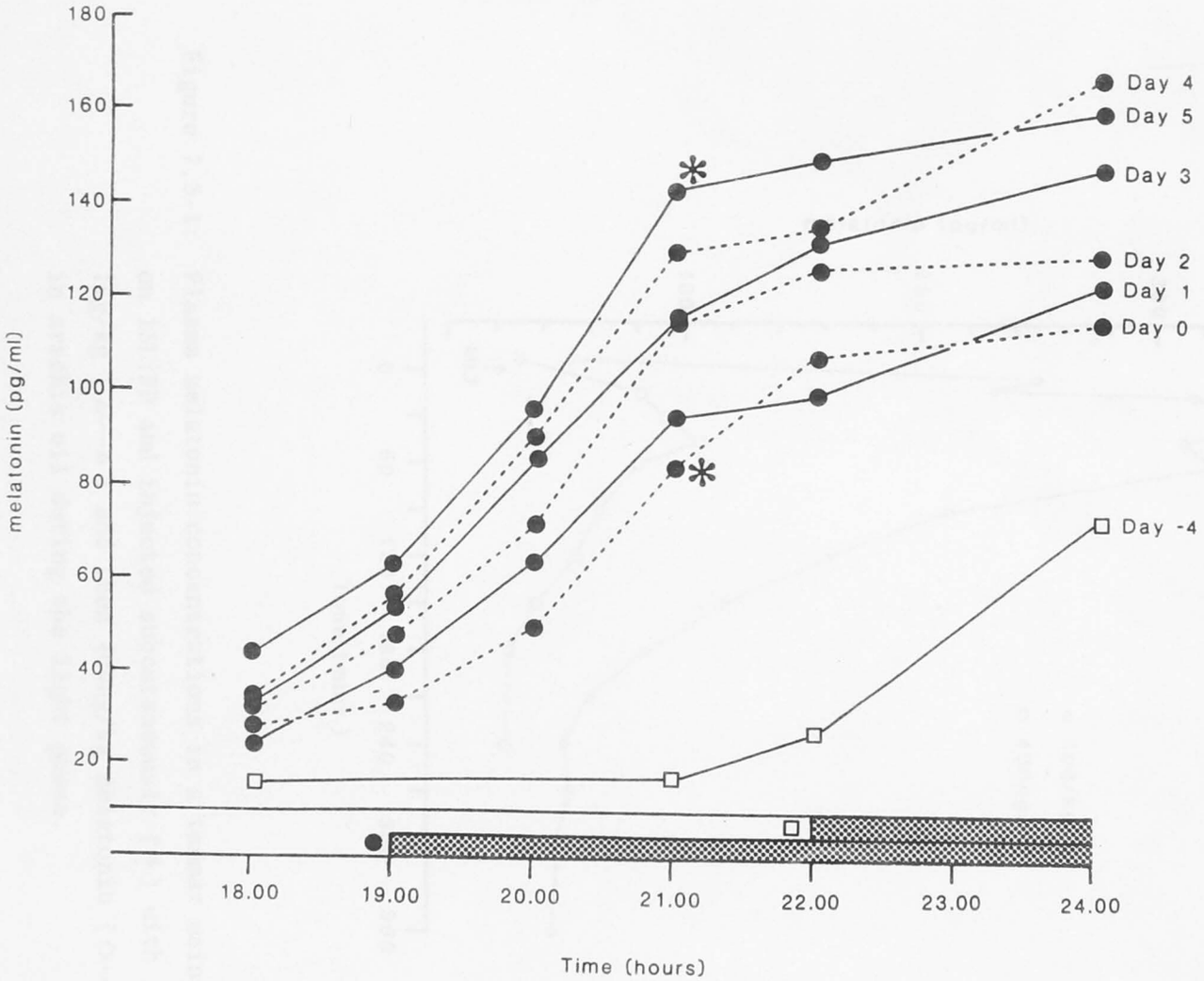

melatonin (pg/ml)
180
140
100
60
20
*
*
*
Day 5
Day 0
18.00
22.00
02.00
06.00
10.00
Time (hours)

Figure 7.2-7: Plasma melatonin concentrations (mean) of four tammars at the onset of the dark phase under 15L:9D (□—□, Day -4) and then 12L:12D (●- - -●, Days 0 to 5). Error bars are omitted for clarity. Stippled bars indicate part of the dark phase of each photoperiod. Note the earlier onset of the nocturnal rise from Day 0 to 5, compared to Day -4, and the progressive daily increase in amplitude. A significant nocturnal rise on Day 0 and Day 5 ($P<0.01$) above the levels at 18.00hrs of the same day, were at 21.00hrs (*). Raw data are given in Appendix C.4 and statistical analyses in Appendices C.5 to C.7.


melatonin (pg/ml)
180
160
140
120
100
80
60
40
20
Day 4
Day 5
Day 3
Day 2
Day 1
Day 0
Day -4
*
*
18.00
19.00
20.00
21.00
22.00
23.00
24.00
Time (hours)

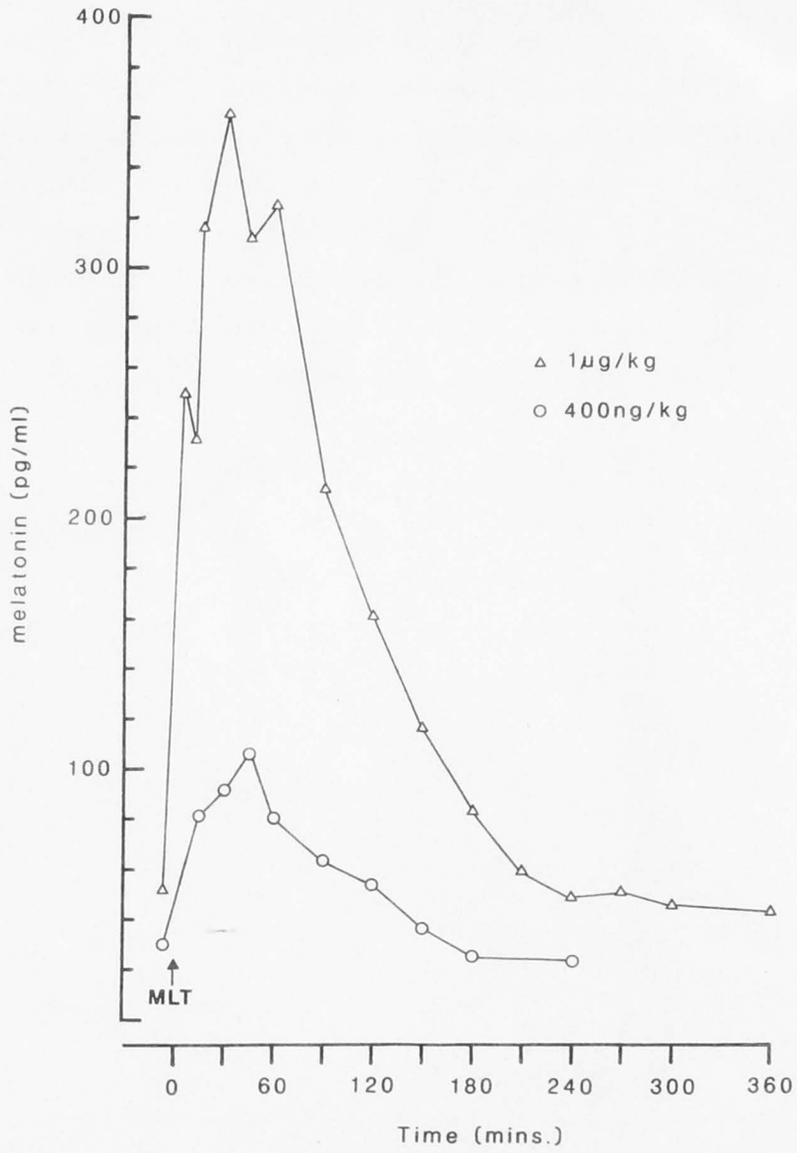


Figure 7.3-1: Plasma melatonin concentrations in a tammar maintained on 15L:9D and injected subcutaneously (↑) with 1µg/kg (△—△) and then 400ng/kg melatonin (○—○) in arachis oil during the light phase.

Figure 7.3-2: Plasma melatonin concentrations in a tammar maintained on 15L:9D (■—■) and after a subcutaneous injection of melatonin (↑ , 400ng/kg) 2.25hrs before dark on the following day (○—○). Note the duration of elevated melatonin is similar to that of the superimposed profile 5 days after the change from 15L:9D to 12L:12D (from Figure 7.2-6). The stippled bars indicate the dark phase of each photoperiod.

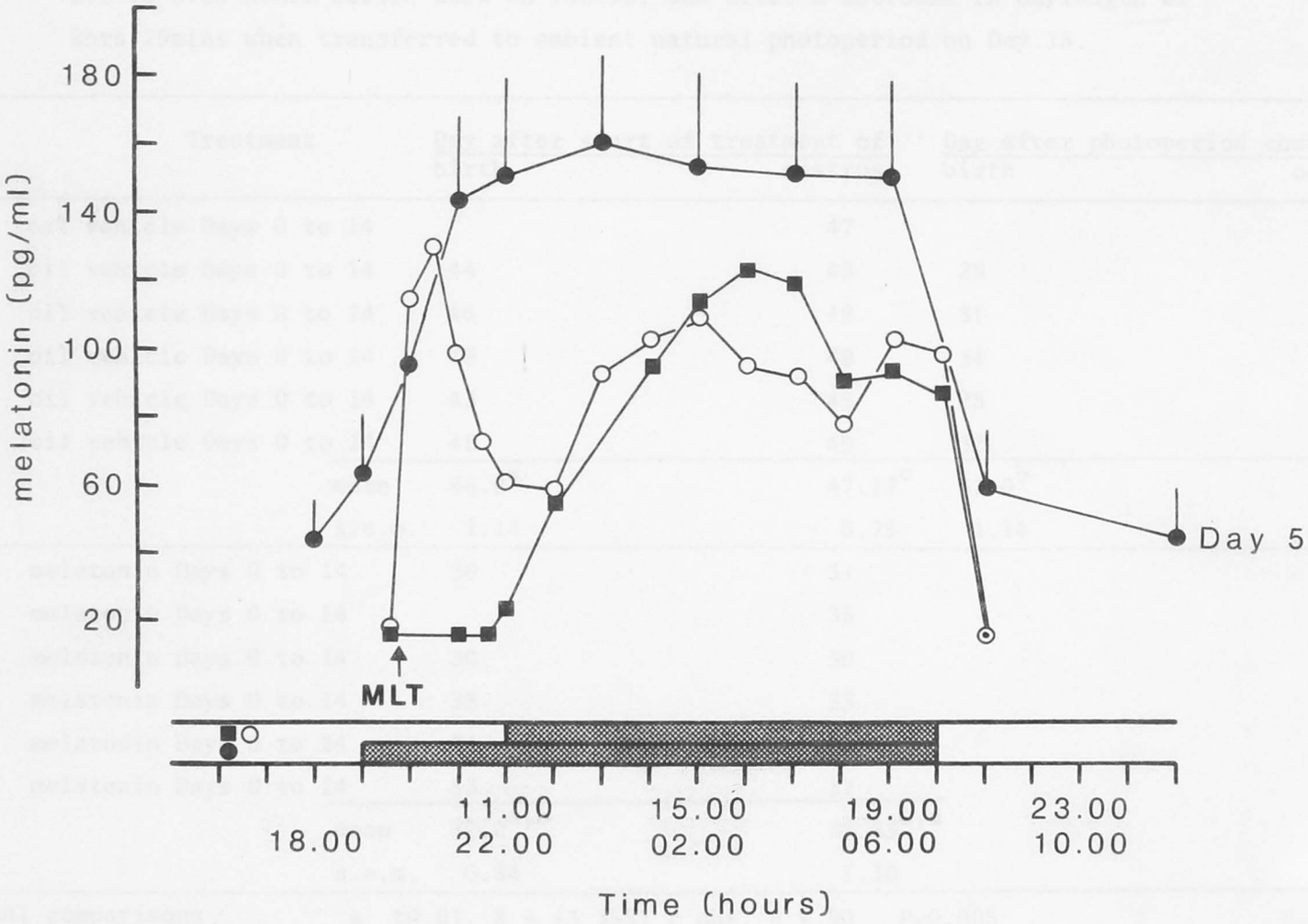

melatonin (pg/ml)
180
140
100
60
20
MLT
Day 5
18.00
11.00
22.00
15.00
02.00
19.00
06.00
23.00
10.00
Time (hours)

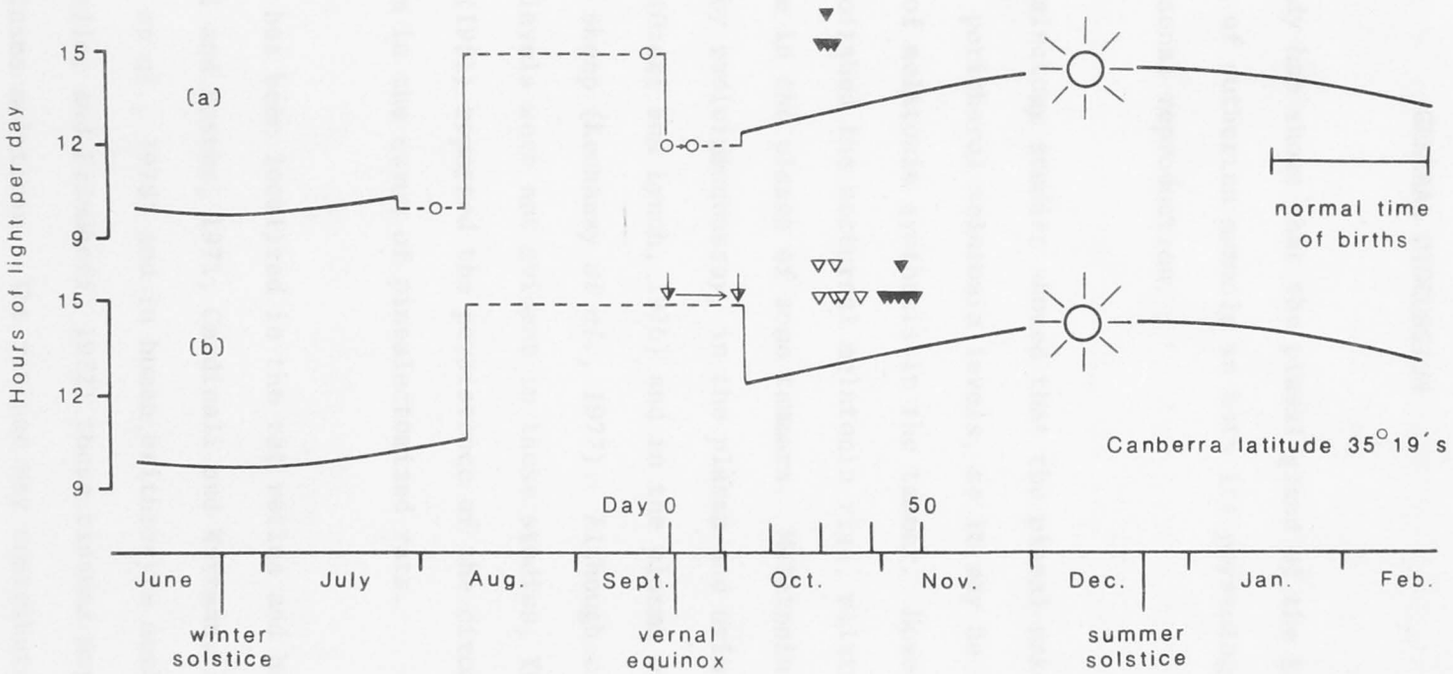
Table 7.3-3: The intervals to birth/oestrus in tammars injected with melatonin or oil vehicle 2.5 to 2.25 hours before dark on 15L:9D, and after a decrease in daylength of 2hrs 29mins when transferred to ambient natural photoperiod on Day 15.

Animal Number	Treatment	Day after start of treatment of birth	Day after start of treatment of oestrus	Day after photoperiod change of birth	Day after photoperiod change of oestrus
395	oil vehicle Days 0 to 14		47		32
696	oil vehicle Days 0 to 14	44	45	29	30
938	oil vehicle Days 0 to 14	46	48	31	33
947	oil vehicle Days 0 to 14	49	49	34	34
982	oil vehicle Days 0 to 14	43	45	28	30
983	oil vehicle Days 0 to 14	48	49	33	34
	mean	46.0[a]	47.17[c]	31.0[b]	32.17[d]
	s.e.m.	1.14	0.75	1.14	0.75
674	melatonin Days 0 to 14	30	31		
965	melatonin Days 0 to 14		38		
4465	melatonin Days 0 to 14	30	30		
4862	melatonin Days 0 to 14	33	33		
5088	melatonin Days 0 to 14	34	34		
5101	melatonin Days 0 to 14	33	37		
	mean	32.0[a,b]	33.83[c,d]		
	s.e.m.	0.84	1.30		

Statistical comparisons (Students t test)

a t0.01, 8 = ±3.355; t obt. = 9.90 P<0.005
b t0.05, 8 = ±2.306; t obt. = -0.71 P>0.05 (n.s.)
c t0.01, 10 = ±3.169; t obt. = 8.88 P<0.005
d t0.05, 10 = ±2,2281 t obt. = -1.11 P>0.05 (n.s.)

Figure 7.3-4: The design and results of the experiments discussed in Chapter 7.2 and 7.3. In experiment (a), 4 tammars gave birth (▼) 31-33 days after a decrease in daylength from 15L:9D to 12L:12D. Blood samples were taken on the days indicated by (○) to measure plasma melatonin (see Chapter 7.2). In experiment (b), 12 tammars were exposed to a similar photoregime except they were maintained on 15L:9D and injected daily with melatonin (400ng/kg) or arachis oil vehicle (↓) 2.5 to 2.25 hours before dark on Days 0 to 14 (Chapter 7.3). This dose, together with the endogenous melatonin, mimicked the increase in duration of the nocturnal rise on Day 5 in Experiment (a) (see Figure 7.3-2). Note the times of birth/oestrus in the melatonin injected group (▽) are the same as in Experiment (a). The control group did not respond to the placebo, but all had a birth/oestrus (▼) after experiencing decreased photoperiod when transferred to outside pens.


Hours of light per day
15
12
9
(a)
15
12
9
(b)
normal time
of births
Canberra latitude 35°19′s
Day 0
50
June
July
Aug.
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
winter
solstice
vernal
equinox
summer
solstice

CHAPTER EIGHT

GENERAL DISCUSSION

This study has shown that the pineal gland of the tammar is similar to that of eutherian mammals in both its physiology, and its function in seasonal reproduction.

The pinealectomy studies showed that the pineal makes the major contribution to peripheral melatonin levels, so it may be considered the major site of melatonin synthesis in the tammar. However, although pinealectomy abolished the nocturnal melatonin rise, melatonin was still detectable in the plasma of some tammars. Melatonin has been measured, also by radioimmunoassay, in the plasma and urine of pinealectomized rats (Ozaki and Lynch, 1976) and in the plasma of long-term pinealectomized sheep (Kennaway *et al.*, 1977). Although circadian changes in the levels were not evident in these studies, Yu, Pang, Tang and Brown (1981) reported the persistence of the circadian melatonin rhythm in the serum of pinealectomized rats.

As HIOMT has been localized in the rat retina and harderian gland (Cardinali and Rosner, 1971; Cardinali and Wurtman, 1972), the mole eye (Pévet *et al.*, 1978) and in human erithrocyte membranes (Rosengarten, Meller and Friedhoff, 1972) these tissues may also contribute to plasma melatonin. The retinae may contribute to the circadian melatonin rhythm, at least in the rat, as a circadian rhythm in retinal melatonin was evident after pinealectomy (Yu, Pang and Tang, 1981). Although melatonin was detectable in the tammar after

pinealectomy, the dark phase levels were typical of those measured during the light phase. Therefore, the extra-pineal source of melatonin is not synthesized in a circadian manner in this species.

As in all other species examined so far, the melatonin levels in the tammar were highest during the dark phase of the daily light/dark cycle. Although not tested directly in this study, exposure to light seems to depress melatonin synthesis, as the levels rose after the onset of dark and declined after the onset of light, and this was observed under various photoperiods. However, the daily light/dark cycle does not appear to be the only factor controlling the daily melatonin rhythm in the tammar.

On the day of a photoperiod change from 15L:9D to 12L:12D, the onset of the nocturnal rise occurred earlier, and during the next five days there was a further daily shift in the onset (Figure 7.2-7). This meant that the full duration of the nocturnal rise under 12L:12D had not occurred until at least five days after the photoperiod change. If, as it seems in the rat (Moore and Klein, 1974), the synthesis of melatonin is stimulated by the SCN, then the shift in the onset may reflect a resynchronization of the endogenous rhythm of the SCN with the onset of the new dark phase of 12L:12D. In this sense, the endogenous rhythm could be regarded as 'free-running' although this term has a more specific meaning in chronobiology i.e. an endogenous rhythm persists in the absence of the zeitgeber, with a phase that approximates that of the exogenous rhythm (Aschoff, 1960); in this study the putative zeitgeber (daily photoperiod) was present. Furthermore, the term 'circadian rhythm'

of plasma melatonin has been used throughout this study, but *in sensu stricto* this may not be correct, as its persistence in constant dark, a criterion for this circadian rhythm, was not established. However, as other aspects of the rhythm appear similar to circadian rhythms of melatonin in other species, it is probably a circadian system in the tammar also.

The superior cervical ganglia (SCG) form part of the retino-pineal pathway in eutherian mammals, and the present study supports the previous findings of Renfree *et al.* (1981), that the SCG are also involved in the control of the pineal of the tammar. In both studies, bilateral ganglionectomy abolished the nocturnal melatonin rise, so the SCG probably mediate the photoperiodic information from the retinae that controls pineal function.

A major finding from this study was that melatonin administration could initiate reactivation of the CL and blastocyst, and so endogenous melatonin may normally be involved in initiation of the breeding season. Changes in the amplitude, duration and phase of the nocturnal melatonin rise have been described in various species, and any of these may be the hormonal signal mediating the effects of photoperiod on reproduction (see Chapter 1). In the tammar, a change in the duration of the nocturnal rise appears to do so, as it does in the ewe (Kennaway *et al.*, 1982; Bittman and Karsch, 1984). After a change from 15L:9D to 12L:12D the duration increased by about 2 hours within 6 days, and this was the period in which the CL reactivated. Melatonin injections given 2.5 to 2.25 hours before dark mimicked this increase in the duration, and also reactivated the CL. The pineal must, therefore, mediate this effect

of decreased daylength by increasing the duration of melatonin synthesis, as it alone produces the nocturnal melatonin rise in the tammar.

The experience of a long duration of the nocturnal melatonin rise does not necessarily lead to reactivation of the CL, however, as the duration of the 10L:14D profile was similar to that of the 12L:12D profile (Figure 7.2-6), but the animals were known to be in seasonal quiescence at this time. There are two possible explanations for this; 1) tammars become refractory to the stimulatory effect of increased duration of melatonin, or 2) it is the shift in onset and/or decline of the melatonin rise, and not the static duration, that is involved.

Refractoriness to photoperiod and melatonin has been described in other species, e.g. the hamster (Reiter, 1981) and Soay ram (Lincoln, 1980). In the tammar, the experience of an increased duration for a certain time may eventually render the target tissues insensitive to the melatonin profile, as proposed for other species (Reiter, 1973), and seasonal quiescence ensues. However, this insensitivity hypothesis is not supported by the findings that, i) some pinealectomized or ganglionectomized tammars still showed seasonal quiescence, and ii) 4 of 7 tammars maintained on 12L:12D from the vernal equinox (September) reactivated after a change to 9L:15D in January (Sadleir and Tyndale-Biscoe, 1977); these animals cannot have been refractory to melatonin although they would have experienced an increased duration of melatonin for a long period.

Alternatively, the actual shift in the onset and/or decline of the melatonin rise, that results in a change in duration, may be the signal for the tammar. At any time during the tammar's breeding season there is a corresponding time in the non-breeding season with an identical photoperiod. The only difference between these times, in terms of photoperiod, is that daylength is decreasing each day during one period, and increasing during the other. As a decrease in daylength can terminate seasonal quiescence (Sadleir and Tyndale-Biscoe, 1977; Hinds and den Ottolander, 1983; Chapter 7.2), and an increase in daylength can initiate seasonal quiescence (Chapter 7.2), it may be the associated increasing and decreasing of the duration of the melatonin rise that are involved, rather than the static duration at any time. These can be regarded as a positive change (increasing duration) or a negative change (decreasing duration). After the summer solstice a positive change is occurring, so seasonal quiescence is abolished, and the breeding season ensues. After the winter solstice a negative change results, so seasonal quiescence is initiated and breeding will no longer occur.

Another explanation for the effects of photoperiod on the tammar could be the phase relationship between some component of the nocturnal melatonin rise and a circadian rhythm in the sensitivity of target tissues. In the hamster there are sensitive periods and only during these times will melatonin injections (Tamarkin *et al.*, 1976; Chen, Brainard and Reiter, 1980), or exposure to light (Stetson, Matt and Watson-Whitmyre, 1976), bring about reproductive changes. If a sensitive period is also present in the tammar the present study has shown that it may occur within two hours before dark in animals

maintained on 15L:9D. Hence melatonin made available during this time, as a result of photoperiod change or melatonin injections, induces reactivation.

Another aspect of particular interest is that the interval to birth/oestrus after melatonin treatment is 6 days longer than that after removal of the pouch young in the breeding season. As it is the pituitary that suppresses the CL and maintains diapause, it must take 6 days for the hypothalamic-pituitary axis to respond to the melatonin signal. The neuroendocrine events that lead to reactivation have yet to be resolved, and an understanding of these events would make the tammar an excellent model in which to study pineal-pituitary relationships, because of the rapid response of the tammar to melatonin, and the identification of a sensitive period to melatonin in the tammar's physiology.

The results of the pinealectomy and ganglionectomy studies have demonstrated that the pineal is also involved in terminating the breeding season of the tammar. Pinealectomy abolished seasonal quiescence in about half of the tammars treated in this study, as they gave birth, or came into oestrus, during the normal seasonal quiescence period. However, seasonal quiescence was retained in the remaining pinealectomized or ganglionectomized tammars as they gave birth, or came into oestrus, at the same time as the controls, after the summer solstice. This result is difficult to reconcile with the findings of Renfree *et al.* (1981), who reported that seasonal quiescence was abolished in all of eight tammars that were ganglionectomized in

lactational quiescence. This cannot have been due to differences between pinealectomy and ganglionectomy, or in the time of year of surgery. In a parallel experiment the effect of either treatment during each half of the year was shown to be the same; seasonal quiescence was only abolished in half of each group. This was a consistent result as the same proportion of pinealectomized tammars retained seasonal quiescence irrespective of the time of year of surgery. An alternative explanation is that, by chance, the animals selected for the study of Renfree *et al.* (1981) were all of the same category, and did not include any animals in which seasonal quiescence is retained in the absence of pineal mediated information.

The persistence of seasonality after pinealectomy could be due to:

1) regrowth of the pineal,
2) non-pineal mediated environmental cues, or
3) an endogenous cue.

Although not tested directly in this study, it is unlikely that regrowth of the pineal would explain the persistence of seasonality, because seasonality was also retained in ganglionectomized animals, in which the pineal could not have been reinnervated from the SCG, as the cell bodies were removed. Furthermore five tammars that had retained seasonal quiescence in the first year failed to do so in the second year (Chapter 6.5), so had actually lost seasonality. This is contrary to what would be expected if the pineal does regrow and reimpose seasonality.

Although pineal mediated photoperiod has been shown to be involved in reproduction of the tammar, non-pineal mediated geophysical cues may also be involved. Temperature, rainfall and nutrition, or the interplay of these, have all been shown to influence reproduction in a variety of mammals, including the larger macropods (Sadleir, 1969), and these may be ancillary cues for the tammar. Furthermore, as two of five pinealectomized tammars responded to a stimulatory photoregimen (Chapter 6.4), the involvement of extra-pineal photosensitivity cannot be dismissed.

Other evidence, however, suggests the involvement of endogenous factors. Three pinealectomized tammars failed to respond to a stimulatory photoregimen in October (15L:9D to 12L:12D), so were presumably no longer photosensitive, and yet they gave birth, or came into oestrus, at the normal time in January whilst maintained on 12L:12D (Chapter 6.4). Furthermore, they subsequently failed to respond to RPY in September, so had again entered seasonal quiescence (Chapter 6.5). Similarly, intact tammars that were maintained on 15L:9D from September gave birth at the same time as the control group in December and January (Sadleir and Tyndale-Biscoe, 1977). These studies show that seasonality is retained in the absence of pineal-mediated information. However, it is unlikely that extra-pineal photosensitivity alone maintained seasonality, as seasonality also persisted in the absence of changing photoperiodic information. Therefore, the most likely explanation for the persistence of seasonality is that an endogenous component is operative. If so, the present study has shown that it can be regarded as a circannual rhythm, as all of the major features of the annual breeding cycle were retained (Chapter 6.5). As discussed in Chapter 1,

the persistence of annual changes in reproductive status after pinealectomy or ganglionectomy have been described in a variety of mammals, and photoperiod may entrain the endogenous circannual rhythm, via the pineal, with seasonal changes in the environment. But if this also occurs in the tammar, why is the circannual rhythm only present in about half of the female population?

Seasonal quiescence may only be a relatively recent acquisition of the island species of macropods (Tyndale-Biscoe, *pers. comm.*). It occurs in *Macropus eugenii* and *M. rufogriseus*, but not in a closely related mainland species, *M. r. banksianus*. These populations have only been separated for the last 10-20,000 years, after the Late Wisconsin glacial melt caused the sea level to rise and isolate the islands of southern Australia (Rawlinson, 1974). Seasonal quiescence may have evolved as an adaptation to the environmental conditions of these islands. Initially, pineal mediated photosensitivity was established in the female population, and this may have led to the acquisition of the endogenous component. However the penetrance of this character in the female population may yet be incomplete. Hence pinealectomy or ganglionectomy abolishes seasonality in those individuals that do not possess this character and rely wholly on pineal mediated photoperiodic information. In those that possess the character, seasonality is retained in the absence of pineal-mediated information. The pineal gland therefore has a predominant role in seasonal reproduction of the female tammar, and together with endogenous information, ensures that the breeding season of the population commences, and is terminated, at the same appropriate time.

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Appendix A. Circadian plasma melatonin levels of PINX and Sham PINX tammars.

Appendix A.1.

Pre-and post-operative levels of plasma melatonin (pg/ml) for tammars subjected to procedures for pinealectomy (PINX) or sham pinealectomy (Sham PINX) in October, 1981. Statistical analyses are given in Appendix A.2.

L = sample taken during light
D = sample taken during dark
* = undetectable, value given is assay sensitivity
- = no sample

Animal No. 560 (failed PINX) is excluded from post-operative analysis.

Appendix A.1.

PRE-OPERATIVE

	Time (hours)							INDIVIDUALS			
								L		D	
Animal No.	16.00	20.00	24.00	04.00	08.00	12.00	16.00				
	L	D	D	D	L	L	L	mean	s.e.m.	mean	s.e.m.
390	*16	23	27	*16	*16	*16	*16	16	0	22	3.21
392	*16	35	65	73	*16	*16	*16	16	0	57.7	11.57
502	*31	72	84	55	*31	*31	*31	31	0	70.3	8.41
503	*16	46	40	26	*16	*16	-	16	0	37.33	5.93
504	*31	33	31	41	*31	*31	-	31	0	35	3.06
505	25	26	42	40	*16	17	*16	18.5	2.18	36	5.03
506	*31	112	97	98	*31	*31	41	33.5	2.5	102.3	4.84
510	*16	58	51	32	*16	34	*16	20.5	4.5	47	7.77
520	*31	82	87	85	43	36	-	36.7	3.48	84.7	1.45
528	53	103	100	125	36	52	48	47.2	3.9	109.3	7.88
547	*16	80	64	67	26	*16	-	19.3	3.33	70.33	4.91
560	*31	70	77	51	*31	*31	*31	31	0	66	7.77
mean	26.08	61.67	63.75	59.08	25.75	27.25	26.88				
GROUP s.e.m.	3.2	8.58	7.37	9.23	2.74	3.26	4.54				

	PINX GROUP							INDIVIDUALS			
								L		D	
Animal No.	16.00	20.00	24.00	04.00	08.00	12.00	16.00				
	L	D	D	D	L	L	L	mean	s.e.m.	mean	s.e.m.
502	*31	*31	*31	*31	*31	*31	*31	31	0	31	0
504	*31	*31	*31	*31	*31	*31	*31	31	0	31	0
506	*31	*31	*31	*31	*31	*31	*31	31	0	31	0
520	37	41	44	42	38	39	72	46.5	8.51	42.3	0.88
528	42	35	37	58	52	50	34	44.5	4.11	43.3	7.36
560	*31	*31	*31	*31	*31	*31	*31	31	0	31	0
mean	34.4	33.8	34.8	38.6	36.6	36.4	39.8				
GROUP s.e.m.	2.23	1.96	2.58	5.3	4.08	3.74	8.07				

Continued....

Appendix A.1 continued.

Animal No.		SHAM PINX GROUP 16.00	20.00	24.00	04.00	08.00	12.00	16.00	INDIVIDUALS L		D	
		L	D	D	D	L	L	L	mean	s.e.m.	mean	s.e.m.
392		33	76	74	36	28	41	30	33	2.86	62	13.01
510		*16	*16	30	36	*16	*16	*16	16	0	27.3	5.93
4700		*16	*16	30	41	*16	*16	*16	16	0	29	7.23
4772		*16	36	51	44	*16	*16	*16	16	0	43.7	4.33
4950		*16	56	154	141	31	23	*16	21.5	3.57	117	30.7
4954		40	29	60	62	25	*16	42	30.7	6.21	50.3	10.68
GROUP	mean	22.83	38.1	66.5	60	22	21.33	22.67				
	s.e.m.	4.42	9.7	18.85	16.67	2.79	4.1	4.49				

Appendix A.2: Statistical comparisons of the mean light and mean dark levels of plasma melatonin (pg/ml) in intact tammars (pre-operative) and tammars subjected to procedures for pinealectomy (PINX) or sham pinealectomy (Sham PINX) in October, 1981. Raw data are given in Appendix A.1. Statistical comparisons were by students t-test for dependent samples.

Pre-operative

Animal Number	LIGHT (mean)	DARK (mean)		DIFFERENCE (d)
390	16	22		-6.0
392	16	57.7		-41.7
502	31	70.3		-39.3
503	16	37.3		-21.3
504	31	35		-4.0
505	18.5	36		-17.5
506	33.5	102.3		-68.8
510	20.5	47		-26.5
520	36.7	84.7		-48.0
528	47.2	109.3		-62.1
547	19.3	70.3		-51.0
560	31	66		-35.0
			$\bar{d}$ =	-35.1
			s.d =	20.74

$$t = \frac{\bar{d} - 0}{sd/\sqrt{n}} = -5.86$$

$t_{.05, 11} = \pm 2.201$, therefore $P < 0.05$

PINX GROUP

502	31	31		0
504	31	31		0
506	31	31		0
520	46.5	42.3		4.2
528	44.5	43.3		1.2
			$\bar{d}$ =	0.9
			s.d =	1.69

$$t = \frac{\bar{d} - 0}{sd/\sqrt{n}} = 1.20$$

$t_{.05, 4} = \pm 2.776$, therefore $P > 0.05$

*No. 560 (failed PINX) is excluded from the analysis

continued...

Appendix A.2 (continued)

Sham PINX GROUP

Animal Number	LIGHT (mean)	DARK (mean)	DIFFERENCE
392	33	62	-29.0
510	16	27.3	-11.3
4700	16	29	-13.0
4772	16	43.7	-27.7
4950	21.5	117	-95.5
4954	30.7	50.3	-19.6
		$\bar{d}$ =	-32.68
		s.d.=	31.62

$$t = \frac{\bar{d} - 0}{sd/\sqrt{n}} = -2.53$$

$t_{.05,\ 5} = \pm 2.571$, therefore $P < 0.05$.

Appendix A.3: Pre-and post-operative levels of plasma melatonin (pg/ml) for tammars subjected to PINX or Sham PINX in May/June, 1982. Statistical analyses are given in Appendix A.4.

L = sample taken during light

D = sample taken during dark

* = undetectable, value given is assay sensitivity

\- = no sample.

? = unable to confirm as no pre-operative rise

Appendix A.3 PRE-OPERATIVE

Animal Number	TIME (Hours) 16.00	20.00	24.00	04.00	18.00	12.00	16.00	20.00	24.00	INDIVIDUALS L mean	L s.e.m.	D mean	D s.e.m.
	L	D	D	D	L	L	L	D	D				
4835	*31	82	95	87	*31	*31	*31	80	105	31.0	0	89.8	4.6
5063	*31	90	87	100	*31	*31	42	58	82	33.8	2.8	84.3	7.0
5161	*31	82	125	105	34	45	49	140	115	39.8	4.3	113.4	9.7
5217	*31	*31	*31	36	*31	*31	*31	*31	*31	31.0	0	32.0	1.0
5218	*31	62	87	105	*31	*31	*31	50	50	31.0	0	70.8	10.9
5226	*31	65	65	76	*31	*31	*31	65	92	31.0	0	72.6	5.3
5234	*16	*16	*16	*16	*16	*16	*16	*16	*16	16.0	0	16.0	0
5238	*16	51	115	110	*16	*16	*16	90	115	16.0	0	96.2	12.2
5316	*16	*16	65	37	*16	*16	*16	35	40	16.0	0	38.6	7.8
5336	*31	78	122	170	*31	*31	*31	96	78	31.0	0	108.8	17.3
5392	*31	45	122	97	*31	*31	*31	78	135	31.0	0	95.4	16.0
mean GROUP	26.9	56.2	84.5	85.4	27.2	28.2	29.5	67.2	78.1				
s.e.m.	2.1	8.0	11.2	13.0	2.2	2.7	3.2	10.6	11.8				
	PINX GROUP												Procedure Confirmed
4835	*31	*31	*31	*31	*31	*31	*31	*31	*31	31.0	0	31.0	0 Yes
5161	50	23	34	40	34	75	75	60	54	58.5	10.1	42.2	6.7 Yes
5217	*31	*31	*31	*31	*31	*31	*31	*31	*31	31.0	0	31.0	0 Yes
5218	*31	*31	*31	*31	*31	*31	*31	*31	*31	31.0	0	31.0	0 Yes
5234	*16	*16	*16	*16	*16	*16	*16	*16	*16	16.0	0	16.0	0 ?
5336	*31	*31	*31	*31	*31	*31	*31	*31	63	31.0	0	37.4	6.4 Yes
mean GROUP	31.7	27.2	29.0	30.0	29.0	35.8	35.8	33.3	37.7				
s.e.m.	4.4	2.6	2.6	3.2	2.6	8.2	8.2	5.9	7.1				

continued....

Appendix A.3 continued

SHAM PINX GROUP

5063	*31	115	94	75	*31	*31	*31	72	125	31.0	0	96.2	10.5	Yes
5226	*31	62	54	34	*31	*31	*31	87	76	31.0	0	62.6	9.1	Yes
5238	*16	72	90	90	*16	*16	*16	*16	72	16.0	0	68.0	13.6	Yes
5316	*16	40	58	*16	*16	*16	*16	58	72	16.0	0	48.8	9.6	Yes
5392	*31	78	78	85	*31	*31	*31	56	*31	31.0	0	65.6	9.9	Yes
mean	25.0	73.4	74.8	60.0	25.0	25.0	25.0	57.8	75.2					
GROUP s.e.m.	3.7	12.2	8.1	14.8	3.7	3.7	3.7	11.8	14.9					

Appendix A.4: Statistical comparisons of the mean light and mean dark levels of plasma melatonin (pg/ml) measured pre-operatively and following PINX or Sham PINX. Raw data are given in Appendix A.3. Statistical comparisons were by students t-test for dependent samples.

PRE-OPERATIVE

Animal Number	LIGHT (mean)	DARK (mean)	DIFFERENCE (d)
4835	31.0	89.8	-58.8
5063	33.8	84.3	-50.5
5161	39.8	113.4	-73.6
5217	31.0	32.0	-1.0
5218	31.0	70.8	-39.8
5226	31.0	72.6	-41.6
5234	16.0	16.0	0
5238	16.0	96.2	-80.2
5316	16.0	38.6	-22.6
5336	31.0	108.8	-77.8
5392	31.0	95.4	-64.4
		$\bar{d}$ =	-46.4
		s.d =	28.7

$$t = \frac{\bar{d} - 0}{sd/\sqrt{n}} = -5.36$$

$t_{0.05, 10} = \pm 2.228$, therefore $P < 0.05$

PINX GROUP

4835	31.0	31.0	0
5161	58.5	42.2	16.3
5217	31.0	31.0	0
5218	31.0	31.0	0
5234	16.0	16.0	0
5336	31.0	37.4	-6.4
		$\bar{d}$ =	1.65
		s.d =	7.65

$$t = \frac{\bar{d} - 0}{sd/\sqrt{n}} = 0.53$$

$t_{.05, 5} = \pm 2.571$, therefore $P > 0.05$

continued....

Appendix A.4 continued

SHAM PINX GROUP

Animal Number	LIGHT (mean)	DARK (mean)	DIFFERENCE (d)
5063	31.0	96.2	-65.2
5226	31.0	62.6	-31.6
5238	16.0	68.0	-52.0
5316	16.0	48.8	-32.8
5392	31.0	65.6	-34.6
		$\bar{d}$ =	-43.2
		s.d =	14.8

$$t = \frac{\bar{d} - 0}{sd/\sqrt{n}} = -6.53$$

$t_{0.05,\ 4} = \pm 2.776$ therefore $P < 0.05$.

Appendix B: Plasma prolactin and progesterone in tammars PINX or Sham PINX in October, 1981.

Appendix B.1: Weekly concentrations of plasma prolactin (ng/ml) in tammars pinealectomized (PINX) or sham operated (Sham) between October 12 - 20, 1981.

* = undetectable, value given is assay sensitivity

** = No. 560 not pinealectomized - excluded from analyses

$P > 0.05$ (students t-test for independent samples), all time point comparisons not significant.

(a) = mean $\pm$ s.e.m. for both PINX and Sham groups measured pre-operatively.

Appendix B.1

Animal Number	Treatment	October 9	October 21	October 28	November 4	November 11	November 18	November 25	December 2	December 9	December 16	December 23	December 30	January 6	January 13	January 20	January 27	February 3	February 11	February 17	February 26
502	PINX	32.6	22.4	19.9	26.8	34.8	22.9	15.7	16.5	32.7	19.8	22.6	10.8	8.3	5.4	15.1	12.9	65			8.6
504	PINX	41.4	75.8	30.2	62.8	31.3	44.2	24.4	58.1	19.9	128	17.5	22.2	17.9	39.7	26.1	41.2	87.5	25.2	30.7	7.5
506	PINX	5.4	*3	6.1	*3	*3	25.4	6.2	33.6	85.8	118.8	56.8	100	21.1	8.6	7.8	56.2	*3			*3
520	PINX	33.2	28.5	18.3	16.1	16	11.4	24.6	11.7	11.7	11.6	18	17.1	63.1	35.9	5.2	19.4	19.3			
528	PINX	40.3	25.2	34.6	45.4	29.7	41.5	24.4	45.3	80.8	20.2	31.1	14.6	19.8	46.2	24.1	54.4	48.6			22.7
560	**	30.4	28.8	12.8	21.4	16.3	34.3	8.6	17.6	6.5	5.2	6.8	8.4	21.6	4.4	4.0	10.2	6.7	4.4	4.0	24
mean			30.9	21.8	30.8	22.9	29.1	19.1	33	46.2	59.7	29.2	32.9	26	27.2	15.7	36.8	34.6	25.2	30.7	10.5
s.e.m.			12.0	4.9	10.6	5.9	6.1	3.6	8.7	15.5	26.1	7.3	16.9	9.5	8.4	4.2	8.9	10.9	0	0	4.3
392	Sham	93.2	48.5	38.6	56.8	84.2	84	88	59.9	55.7	41	49	77.1	50.6	37	40.5	33	49	41	64.9	10.9
510	Sham	57.2	66.2	48.7	53.2	44.6	46	54.1	57.1	41.7	64.1	79.9	71.3	67	70.7	65.3	60.6	63	50.7	48.2	30.9
4700	Sham			21.6	35.4	33.9	20.3	33.6	29	23.4	8.6	12.2	23.9	56.3	13	8.4	12	97.2			
4772	Sham			24.5	44.4	62.6	51.7	70	67.1	63.5	60.2	54.4	54.6	48.1	50.3	37.7	19.4	22	21.6	14.5	
4950	Sham		8.4	18.9	35.8	25.8	19.7	20.7	20.1	17.1	15.6	13.5	30.7	21	32	45.7	10.4	15.1	67.8	30.2	12.8
4954	Sham		19.5	29.7	50.1	17.7	14.5	9.9	13.5	4.1	6.5	35.7	23	19.2	15.7	11.7	28.2	14.4	18.4	14.3	8.9
mean	(a)	43.3	35.6	30.3	45.9	44.8	39.4	46.1	41.1	34.3	32.7	40.8	46.8	43.7	36.5	34.9	27.3	43.5	39.9	34.4	15.9
s.e.m.		10.18	13.2	4.6	3.67	10.1	10.9	12.3	9.4	9.5	10.6	10.6	9.9	7.9	8.9	8.8	7.6	13.4	9.2	9.8	5.1

Appendix B.2: Pre-and post-operative levels of plasma prolactin (ng/ml) for tammars pinealectomized (PINX) or sham operated (Sham PINX) between October 12-20, 1981.

L = sample taken during light

D = sample taken during dark

* = undetectable, value given is assay sensitivity

- = no sample.

Appendix B.2

PRE-OPERATIVE

Time (hours)

Animal Number	16.00	20.00	24.00	04.00	08.00	12.00	16.00	INDIVIDUALS	
	L	D	D	D	L	L	L	mean	s.e.m.
390	19.8	12.5	12.4	17.8	22.4	10.5	14.3	15.7	1.66
392	31.2	24.3	22.0	20.2	55.2	56.7	50	37.1	6.15
502	35.1	27.5	8.7	10.3	27.4	10.8	14.8	19.2	3.99
503	*3.0	*3.0	*3.0	6.8	18.5	*3.0	3.4	5.8	2.18
504	24.9	3.1	*3.0	*3.0	28.9	*3.0	-	11.0	5.06
505	25.1	25.8	31.9	28.0	42.0	37.2	42.2	33.2	2.78
506	11.7	4.7	*3.0	4.3	7.3	*3.0	11.0	6.4	1.38
510	36.0	35.3	28.3	31.6	36.1	32.1	31.9	33.0	1.09
520	23.0	14.9	13.5	13.2	21.9	11.9	16.1	16.4	1.65
528	15.0	10.5	18.7	32.7	18.5	25.4	18.9	20.0	2.72
547	39.5	35.6	33.9	18.7	43.8	27.4	22.3	31.6	3.46
560	31.1	29.7	28.8	25.2	41.6	28.2	26.7	30.2	2.04
mean GROUP	24.62	18.91	17.27	17.65	30.3	20.77	22.87		
s.e.m.	3.14	3.53	3.36	2.99	3.94	4.80	4.18		
				PINX GROUP					
502	12.1	7.7	8.3	8.1	12.4	19.1	8.0	10.8	1.57
504	15.0	10.9	14.4	12.4	26.8	40.1	14.3	19.1	4.01
506	*3.0	*3.0	24.8	*3.0	*3.0	*3.0	*3.0	6.1	3.11
520	10.7	7.3	7.5	8.0	9.4	8.2	6.5	8.2	0.53
528	18.7	11.1	16.1	11.4	17.2	30.5	13.6	16.9	2.51
560	14.4	6.0	4.2	41.5	5.3	13.1	8.5	13.3	4.93
mean GROUP	11.90	8.0	14.22	8.58	13.76	20.18	9.08		
s.e.m.	2.61	1.48	3.13	1.65	3.99	6.87	2.15		

continued....

Appendix B.2 continued

SHAM PINX GROUP

	16.00	20.00	24.00	04.00	08.00	12.00	16.00	INDIVIDUALS	
	L	D	D	D	L	L	L	mean	s.e.m.
392	88.2	40.7	42.2	105	43.2	89.8	57.4	66.6	10.21
510	40.7	30.7	31.7	39.9	33.2	44.1	37.4	36.8	1.92
4700	20.3	26.0	21.6	95.6	16.7	33.3	48.0	37.4	10.49
4772	51.7	41.4	43.5	53.8	45.6	48.8	49.3	47.7	1.68
4950	19.7	16.3	18.4	18.0	16.0	12.8	26.9	18.3	1.66
4954	14.5	10.8	9.3	30.4	12.9	32.2	29.4	19.9	3.86
mean GROUP	39.28	27.65	27.78	57.12	27.93	43.5	41.42		
s.e.m.	11.5	5.11	5.59	14.52	5.97	10.56	4.94		

Appendix B.3: Analysis of variance of the circadian prolactin profile of tammars PINX or Sham PINX in October, 1981. Values given are the daily means (ng/ml).

	TREATMENT	
Pre operative (1)	PINX (2)	Sham PINX (3)
15.7	10.8	66.6
37.1	19.1	36.8
19.2	6.1	37.4
5.8	8.2	47.7
11.0	16.9	18.3
33.2		19.9
6.4		
33.0		
16.4		
20.0		
31.6		
30.2		
Tj 259.6	61.1	226.7
$\bar{T}j$ 21.63	12.22	37.78

ANOVA TABLE

Source	d.f.	SS	MS	F
Treatment	2	1,900.01	950.01	6.14
Error	20	3,096.07	154.8	
Total	22	4,996.08		

F .05; 2,20 = 5.85, therefore P < 0.05

Appendix B.4: *A posteriori* comparisons for ANOVA in Appendix B.3.

Treatments ($\bar{T}$) are: 1 pre-operative
2 pinealectomized
3 sham pinealectomized

Values are differences between means. Newman-Keuls critical values are given in parentheses.

	$\bar{T}_2$	$\bar{T}_1$	$\bar{T}_3$
$\bar{T}_2$		9.41 (14.21)	*25.56 (17.24)
$\bar{T}_1$		-	*16.15 (14.21)
$\bar{T}_3$			-

*Significant difference between treatments at $P < 0.05$ (see Mendenhall and Ramey, 1973, p.244 for test outline).

Appendix B.5: Weekly concentrations of plasma progesterone (pg/ml) in tammars PINX or Sham PINX between October 12-20, 1981

PINEALECTOMY GROUP

	Animal Number					
DATE	502	504	506	520	528	560
9.10.81	117	163	141	151	129	194
21.10.81	94	148	123	150	148	180
28.10.81	96	133	111	131	133	160
4.11.81	126	156	77	227	114	189
11.11.81	111	115	117	215	106	200
18.11.81	97	104	110	368	101	168
25.11.81	110	113	109	226	120	133
2.12.81	107	147	87	270	179	231
9.12.81	132	101	115	194	141	122
16.12.81	117	168	81	156	236	142
23.12.81	114	199	110	101	225	88
30.12.81	167	250	177	188	211	149
6.1.82	469	234	189	184	206	201
13.1.82	291	190	396	220	151	285
20.1.82	94	205	423	533	265	231
27.1.82	169	230	230	144	141	131
3.2.82	102	190	190	227	143	108
11.2.82		122				141
17.2.82						305
24.2.82						299
26.2.82	287	180	256	265	286	812

SHAM GROUP

DATE	392	510	4700	4772	4950	4954
9.10.81	277	117				
16.10.81			143	246	234	153
21.10.81	147	41				
23.10.81			137	225	172	153
28.10.81	189	98	91	213	151	93
4.11.81	203	69	144	103	170	92
11.11.81	196	88	91	78	129	83
18.11.81	212	95	108	105	161	81
25.11.81	137	42	151	221	227	21
2.12.81	200	50	101	162	136	116
9.12.81	156	49	148	186	165	99
16.12.81	165	85	126	134	126	122
23.12.81	161	107	126	143	102	155
30.12.81	192	192	137	264	172	613
6.1.82	184	148	171	206	214	192
13.1.82	334	317	143	393	254	397
20.1.82	502	545	302	435	397	461

continued....

Appendix B.5 (continued).

Date	392	510	4700	4772	4950	4954
27.1.82	335	116	245	346	129	150
3.2.82	152	288	160	190	224	183
11.2.82	342	232		228	528	362
17.2.82	214	297		434	369	321
24.2.82		154				
25.2.82			159	552		150
26.2.82	475	106			228	202

Appendix C: The circadian plasma melatonin profile of intact tammars under various photoregimen.

C.1: Plasma melatonin concentration for individual tammars exposed to winter, spring, summer and autumn photoperiods.

C.2: Analysis of variance of the circadian melatonin profiles in each season.

C.3: Statistical comparisons of each time point for each season for the data given in Appendix C.1.

C.4: Plasma melatonin concentrations (pg/ml) for individual tammars exposed to three photoperiods (10L:14D, 15L:9D and 12L:12D).

C.5: Analysis of variance of the circadian melatonin profiles of tammars exposed to photoperiods of 10L:14D, 15L:9D and 12L:12D.

C.6: Statistical comparison of each time point under each photoperiod for the data given in Appendix C.4.

C.7: Statistical comparisons between light and subsequent time points during the dark phase on Day 0 to Day 5.

Appendix C.1: Plasma melatonin concentrations (pg/ml) for individual tammars exposed to winter, spring, summer and autumn photoperiods. The season and times of sampling are indicated. L = sample taken during light; D = sample taken during dark, - no sample taken; * = undetectable, value given is assay sensitivity. Statistical analyses are given in Appendix C.2.

		TIME (hours)														
	Animal	16.00	18.00	20.00	22.00	24.00	02.00	04.00	06.00	08.00	10.00	12.00	14.00	16.00	18.00	20.00
	Number	L	D	D	D	D	D	D	D	L	L	L	L	L	D	D
WINTER (JUNE)	639	*16	47	-	64	69	-	-	-	*16	*16	*16	*16	*16	45	80
	675	*16	56	104	94	110	104	87	87	*16	*16	*16	*16	*16	43	43
	679	*16	39	78	44	75	-	83	-	*16	*16	*16	*16	*16	38	35
	682	*16	52	86	72	99	140	118	128	*31	*16	*16	*16	*16	24	70
	684	*31	38	90	95	86	90	80	86	*31	*31	*31	*31	*31	48	74
	688	29	55	92	115	120	112	91	117	32	29	22	22	25	50	91
	mean	20.7	47.8	90	80.7	93.17	111.5	91.8	104.5	23.67	20.67	19.5	19.5	20	41.3	65.5
	s.e.m.	2.96	3.22	4.24	10.42	8.17	10.53	6.81	10.63	3.43	2.96	2.50	2.50	2.65	3.86	8.92
		L	D	D	D	D	D	D	L	L	L	L	L	L	D	D
SPRING (OCTOBER)	639	*16	*16	78	110	112	122	94	33	5	12	16	9	65	53	144
	675	35	*31	94	110	140	146	81	6	15	25	18	13	32	38	126
	679	*16	*16	75	89	110	140	77	42	38	21	19	30	25	70	124
	682	*16	*16	110	160	155	160	88	*16	4	*16	8	24	*16	40	136
	684	*31	*31	115	130	179	162	178	44	*31	44	*31	*31	*31	*31	105
	688	26	51	150	163	180	197	215	125	58	47	51	54	71	85	155
	mean	23.33	26.83	103.7	127	146	154.5	122.2	44.3	25.2	25.5	23.8	26.8	40	52.8	131.7
	s.e.m.	3.48	5.42	11.39	12.13	12.67	10.38	24.1	17.24	8.63	5.12	6.22	6.54	9.19	8.55	7.11
		L	L	D	D	D	D	D	L	L	L	L	L	L	L	D
SUMMER (JANUARY)	639	-	34	101	181	209	189	219	129	-	49	-	37	-	-	99
	675	-	19	96	216	251	271	271	131	-	4	-	46	-	20	91
	679	-	37	94	184	234	229	249	174	-	37	-	29	-	32	114
	682	-	12	85	240	320	325	380	165	-	42	-	35	-	*16	110
	684	-	*31	76	152	240	215	188	62	-	*31	-	*31	-	*31	62
	688	-	147	112	248	250	225	250	210	-	62	-	54	-	62	86
	mean		46.7	94	203.5	250.7	242.3	259.5	145.2		37.5		38.7		32.2	93.7
	s.e.m.		20.44	5.11	15.28	15.2	19.77	26.8	20.66		7.00		3.90		7.36	7.7
		L	D	D	D	D	D	D	D	L	L	L	L	L	L	D
AUTUMN (APRIL)	639	41	42	149	169	229	209	259	214	54	-	30	-	55	-	144
	675	32	50	216	256	251	271	276	316	31	-	9	-	31	-	236
	679	26	22	174	199	249	274	209	254	84	-	32	-	22	-	199
	682	30	57	190	250	360	430	540	435	47	-	22	-	10	-	400
	684	*31	*31	125	105	143	172	170	168	*31	-	*31	-	*31	-	162
	688	56	66	234	200	280	232	268	250	165	-	47	-	49	-	200
	mean	36	44.7	181.3	196.5	252	264.7	287	272.8	68.7		28.7		33		223.5
	s.e.m.	4.48	6.69	16.66	22.77	28.79	36.61	53.24	38.07	20.85		5.14		6.83		37.67

Appendix C.2: Analysis of variance of the circadian melatonin profiles of six tammars under winter, spring, summer and autumn photoperiods.

Source of Variation	Degrees of Freedom	Variance Ratio	Significance Level
time	14	134.714	$P < 0.01$
animal	5	20.173	$P < 0.01$
photoperiod	3	84.503	$P < 0.01$
time x animal	70	2.153	$P < 0.01$
time x photo-period	35	5.952	$P < 0.01$
animal x photo-period	15	4.292	$P < 0.01$

Appendix C.3: Statistical comparison of the circadian melatonin profile at each time point during each season (designated as A, B, C or D) using the Least Significant Difference, calculated from S.E.D. times students t (Bliss, 1967, p.252). At each time, significant differences ($P < 0.05$) between one season and another are indicated by the letter beneath each season. For example, at 06.00hrs, the levels in Winter (A) are significantly different ($P < 0.05$) from both the Spring (B) and Autumn (D) levels. However, the Summer (C) levels are only significantly different from Spring (B).

N.S. - no significant differences ($P > 0.05$). The analyses were adjusted for missing values. Raw data are given in Appendix C.1.

TIME (hours)	WINTER (A □)	SPRING (B ●)	SUMMER (C ■)	AUTUMN (D ○)
16.00	D	D	N.S.	A, B
18.00	B	A	N.S.	N.S.
20.00	D	D	D	A, B, C
22.00	B, C, D	A, C, D	A, B	A, B
24.00	B, C, D	A, C, D	A, B	A, B
02.00	B, C, D	A, C, D	A, B	A, B
04.00	C, D	C, D	A, B	A, B
06.00	B, D	A, C, D	B	A, B
08.00	D	D	N.S.	A, B
10.00	N.S.	N.S.	N.S.	N.S.
12.00	N.S.	N.S.	N.S.	N.S.
14.00	C	C	A, B	N.S.
16.00	B	A	N.S.	N.S.
18.00	N.S.	C	B	N.S.
20.00	B, D	A, C, D	B, D	A, B, C

Appendix C.4: Plasma melatonin concentrations (pg/ml) for individual tammars exposed to three photoperiods (10L:14D, 15L:9D and 12L:12D). The days and times of sampling are indicated.

L - sample taken during light

D - sample taken during dark

\- - no sample taken

* - undetectable, value given is assay sensitivity.

Statistical analyses are given in Appendices C.5, C.6 and C.7.

Appendix C.4

	Animal Number	TIME (hours) 16.00	18.00	19.00	20.00	21.00	22.00	24.00	02.00	04.00	06.00	08.00	12.00
		L	D	D	D	D	D	D	D	D	D	L	L
10L:14D (Day -46)	5055	*16	36		73		73	120	64	73	92	*16	*16
	5374	*16	19		54		36	33	30	28	16	*16	*16
	5380	*16	*16		27		28	47	41	51	38	*16	*16
	5704	*16	53		84		104	106	98	81	33	*16	*16
	mean	16	31		59.5		60.3	76.5	58.3	58.3	44.8	16	16
	s.e.m.	0	8.6		12.5		17.6	21.5	15	11.9	16.4	0	0
			L			L	D	D	D	D	D	L	L
15L:9D (Day -4)	5055		*16			20	*16	120	135	150	150	22	*16
	5374		*16			*16	*16	44	43	42	60	33	33
	5380		*16			*16	62	-	64	62	62	45	*16
	5704		*16			*16	*16	58	92	109	105	*16	*16
	mean		16			17	27.5	74	83.5	90.8	94.3	29	20.5
	s.e.m.		0			0.7	11.5	23.4	19.9	24.2	21.3	6.4	4.5
			L	D	D	D	D	D	D	D	D	L	L
12L:12D (Day 0)	5055		*16	*16	*16	51	58	73	64	73	92	*16	*16
	5374		30	32	58	78	86	95	83	104	78	31	27
	5380		25	37	44	82	102	109	110	86	79	56	21
	5704		39	47	82	127	190	190	90	180	165	74	38
	mean		27.5	33	50	84.5	109	117	86.8	111	103.5	44.3	25.5
	s.e.m.		4.8	6.5	13.8	15.8	28.5	25.5	9.5	23.9	20.7	12.9	4.7
			L	D	D	D	D	D					
12L:12D (Day 1)	5055		*16	*16	*16	46	51	92					
	5374		-	41	63	80	65	100					
	5380		23	40	59	96	112	135					
	5704		33	66	118	158	173	173					
	mean		24	40.8	64	95	100.3	125					
	s.e.m.		4.9	10.2	20.9	23.4	27.5	37					
			L	D	D	D	D	D					
12L:12D (Day 2)	5055		*16	*16	18	64	82	105					
	5374		27	51	62	74	73	65					
	5380		25	44	82	150	175	157					
	5704		60	81	125	173	181	198					
	mean		32	48	71.8	115.3	127.8	131.3					
	s.e.m.		9.6	13.3	22.2	27.2	29.1	29.2					
			L	D	D	D	D	D					
12L:12D (Day 3)	5055		*16	*16	46	51	82	105					
	5374		37	48	69	74	71	81					
	5380		29	47	89	168	185	220					
	5704		47	103	138	173	191	191					
	mean		32.3	53.5	85.5	116.5	132.3	149.3					
	s.e.m.		6.5	18.1	19.6	31.3	32.3	33.3					
			L	D	D	D	D	D					
12L:12D (Day 4)	5055		*16	*16	36	92	82	120					
	5374		36	68	110	88	110	117					
	5380		30	55	100	175	210	205					
	5704		55	85	116	167	143	232					
	mean		34.3	56	90.5	130.5	136.3	168.5					
	s.e.m.		8.1	14.7	18.5	23.5	27.6	29.4					
			L	D	D	D	D	D	D	D	D	L	L
12L:12D (Day 5)	5055		*16	18	40	105	92	135	120	135	120	25	*16
	5374		37	59	79	102	109	111	104	106	100	44	51
	5380		52	78	115	162	170	168	160	140	158	79	47
	5704		72	98	152	208	230	232	232	230	230	98	74
	mean		44.3	63.3	96.5	144.3	150.3	161.5	154	152.8	152	61.5	47
	s.e.m.		11.8	17.1	24	25.3	31.4	26.2	28.5	26.8	28.6	16.5	11.9

Appendix C.5: Analysis of variance of the circadian melatonin profiles of tammars exposed to photoperiods of 10L:14D, 15L:9D and 12L:12D.

Source of Variation	Degrees of Freedom	Variance Ratio	Significance Level
time	10	73.229	$P < 0.001$
animal	3	38.987	$P < 0.001$
photoperiod	3	83.318	$P < 0.001$
time x animal	30	1.614	n.s.
time x photoperiod	26	6.414	$P < 0.001$
animal x photo-period	9	15.035	$P < 0.001$

Appendix C.6: Statistical comparison of the plasma melatonin concentrations at each time point during a stimulatory photoregimen. Comparisons were made using the Least Significant Difference method (Bliss, 1967, p.252). At each time, a significant difference ($P < 0.05$) between one photoperiod and another is indicated by the letter beneath each photoperiod. For example, at 04.00hrs the 10L:14D level is significantly different from that of 15L:9D (B) and 12L:12D (Day 5) (D), whereas the 12L:12D (Day 0) level is not significantly different from any other photoperiod (N.S., $P > 0.05$). The analyses were adjusted for missing values. Raw data are given in Appendix C.1.

	PHOTOPERIOD			
Time (hours)	10L:14D (A □)	15L:9D (B ■)	12L:12D (Day 0) (C ●)	12L:12D (Day 5) (D ●)
18.00	N.S.	N.S.	N.S.	N.S.
19.00	N.S.	N.S.	N.S.	N.S.
20.00	N.S.	N.S.	N.S.	N.S.
21.00	B, D	A, C, D	B, D	A, B, C
22.00	D,	C, D	B,	A, B,
24.00	D,	N.S.	N.S.	A,
02.00	D,	N.S.	D	A,
04.00	B, D,	A,	N.S.	A,
06.00	B, D,	A,	N.S.	A,
08.00	D,	N.S.	N.S.	A,
12.00	D,	D,	N.S.	A, B,

Appendix C.7: Statistical comparisons of plasma melatonin levels (pg/ml) between 18.00 hrs (light) and subsequent time points during the dark phase to determine the onset of the nocturnal rise on Day 0 and Day 5 of 12L:12D. Comparisons are made using students t-test for dependent samples with adjustments (dividing an appropriate α level by number of comparisons) for multiple t-tests. Raw data are given in Appendix C.3.

12L:12D (Day 0)

LIGHT	c.f.	DARK		
18.00hrs		19.00hrs	20.00hrs	21.00hrs
t 0.01,3 =		-4.541	-4.541	-4.541
t obt. =		-2.00	-2.50	-5.05
P =		>0.01	>0.01	<0.01

12L:12D (Day 5)

LIGHT	c.f.	DARK		
18.00hrs		19.00hrs	20.00hrs	21.00hrs
t 0.01, 3 =		-4.541	-4.541	-4.541
t obt. =		-3.31	-4.28	-6.62
P =		>0.01	>0.01	<0.01

Appendix D: The dates of birth/oestrus in animals used in the experiments of Chapter 6.5.

D.1: The dates of birth/oestrus in pinealectomized, ganglionectomized or sham operated tammars after RPY in March and September in the year after surgery.

RPY - removal of pouch young
PE - not carrying pouch young
* - not RPY
** - no birth/oestrus throughout study
~ - estimated dates
† - animal died

Appendix D.1

GROUP 2

Animal Number	Treatment May 31 - June 3 1982	Treatment March 28 (1983)	Date of Birth	Date of Oestrus	Days after RPY	Treatment Sept. 21 (1983)	Date of Birth	Date of Oestrus	Days after RPY
5063	Sham PINX	RPY	-30.5.83		63	RPY	-28.1.84		129
5226	Sham PINX	RPY	-30.4.83		33	RPY	- 6.3.84		164
5238	Sham PINX	RPY	-24.4.83		27	RPY†			
5316	Sham PINX	RPY	- 3.6.83		67	RPY	-29.1.84		130
5392	Sham PINX	RPY	-30.4.83		33	RPY	-31.1.84		132
4835	PINX	RPY	- 3.5.83		36	RPY	19.10.84		28
5161	PINX	RPY	-27.4.83		30	RPY	10.10.84		28
5217	PINX	RPY	-11.5.83		44	RPY	- 2.2.84		134
5218	PINX	RPY	-30.4.83		33	RPY	- 1.2.84		133
5234	PINX	RPY	-29.4.83		32	RPY	-15.2.84		147
5536	PINX	PE				PE			

GROUP 3

Animal Number	Treatment Oct. 18-28 (1982)	Treatment March 28 (1983)	Date of Birth/Oestrus	Days after RPY	Treatment Sept. 21 (1983)	Date of Birth	Date of Oestrus	Days after RPY
526	Sham SCGX	RPY	-25.4.83	28	RPY	-29.2.84		161
618	Sham SCGX	RPY	-26.4.83	29	†			
641	Sham SCGX	RPY	-26.4.83	29	PE	-27.1.84		*
693	Sham SCGX	RPY	-24.4.83	27	RPY	- 4.2.84		136
5597	Sham SCGX	RPY	-24.4.83	27	RPY	-27.1.84		128
617	SCGX	RPY			PE		21.3.84	*
654	SCGX	RPY	-23.7.83	117	RPY	-31.1.84		132
4670	SCGX	RPY	-26.4.83	29	RPY	19.10.83		28
5085	SCGX	RPY	-30.4.83	33	RPY	-26.10.83		35
637	SCGX	RPY	-22.8.83	147	RPY	-14.1.84		115
658	SCGX	RPY	- 3.5.83	36	RPY†			
694	SCGX	PE			PE			**

continued

Appendix D.1 (continued)

GROUP 4

Animal Number	Treatment	Pouch Young Lost	RPY	Date of Birth	Days after RPY
4948	Sham PINX		21.9.83	26.2.84	158
5279	Sham PINX		21.9.83	23.1.84	124
5295	Sham PINX		21.9.83	27.2.84	128
5298	Sham PINX		21.9.83	2.2.84	134
5401	Sham PINX	12.8→20.9.83		1.2.84	*
5278	PINX †				
5282	PINX		21.9.83	24.2.84	156
5317	PINX	**			
5347	PINX †				
5358	PINX	12.8→20.9.83		2.2.84	*
5427	PINX		21.9.83 †	(no birth by 3.1.84)	

GROUP 5

Animal Number	Treatment Dec. 6-8 (1982)	Treatment March 28 (1983)	Date of Birth/Oestrus	Days after RPY	Treatment Sept. 21 (1983)	Date of Birth	Date of Oestrus	Days after RPY
4922	Sham PINX	RPY	- 23.4.83	26	RPY	- 5.2.84		137
4947	Sham PINX	RPY †						
5201	Sham PINX	RPY †						
5385	Sham PINX	RPY	- 23.4.83	26	RPY	- 7.2.84		139
699	PINX	RPY	- 26.5.83	59	RPY	19.10.83		28
4836	PINX	RPY	- 17.7.83	111	RPY †			
4873	PINX	RPY	- 26.4.83	29	RPY	- 3.2.84		135
4968	PINX	RPY	- 10.5.83	43	RPY	- 26.10.83		35
5304	PINX	RPY	- 31.5.83	64	RPY	- 6.2.84		138
5341	PINX	RPY	- 2.5.83	35	RPY	- 3.12.83		73

Appendix E: Publications arising from the work presented in this thesis.

E.1: McConnell, S.J., and Hinds, L.A. (1982). The role of the pineal gland in seasonal quiescence in the tammar wallaby. *Proc. Aust. Soc. Reprod. Biol.* 14, 43 (abstract)

E.2: McConnell, S.J. (1983). Melatonin profile changes in the tammar wallaby. *Proc. Int. Union Physiol. Sci.* 15, 438.14 (abstract).

E.3: McConnell, S.J. (1983). Comparison of the effects of superior cervical ganglionectomy and pinealectomy on seasonal quiescence in the tammar wallaby. *Proc. Aust. Soc. Reprod. Biol.* 15, 67 (abstract).

E.4: McConnell, S.J. (1983). Pineal gland function in the tammar wallaby. *Bull. Aust. Mamm. Soc.* 8, 25 (Bolliger Award Paper) (abstract).

THE ROLE OF THE PINEAL GLAND IN SEASONAL QUIESCENCE IN THE TAMMAR WALLABY

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The female tammar wallaby is a highly seasonal breeder that exhibits embryonic diapause which is due to a pituitary-induced suppression of the CL (1,2). From January to June removal of the pouch young (RPY) leads to reactivation of the quiescent corpus luteum (CL) and blastocyst (lactational quiescence, LQ) but RPY from July to December is ineffective (seasonal quiescence, SQ). Although the environmental factors responsible for the initiation and maintenance of SQ are not known, photoperiod has been implicated (3), as has the pineal gland since superior cervical ganglionectomy when performed in LQ presented SQ (4).

This study examined the possible role of the pineal gland in maintaining SQ. Removal of the pineal was expected to induce CL reactivation prior to the onset of breeding around the summer solstice.

A method for pinealectomy (Px) in the tammar was developed. Using a mid-dorsal interhemispheric approach the pineal was visualized and either aspirated (Px; n=6) or left intact (Sham; n=6). The completeness of Px was confirmed by histological examination of the epithalamic region. Four-hourly diurnal bleeds were taken pre- and post-operatively, and plasma melatonin was determined using the radioimmunoassay of Kennaway et al. (5) which was validated in this study for the tammar. The operations were performed in mid-October 1981, and thereafter regular checks were made until birth or mating was detected.

Five of six females were successfully pinealectomized. The preoperative diurnal profile in all 12 animals showed a significant nocturnal rise in melatonin. After surgery no rise was observed in the 5 Px animals but was seen in all Shams. The time from surgery to reactivation was not significantly different between the Px and Sham groups; all animals reactivated around the normal time in December-January.

The results suggest that either (a) the pineal is not involved in maintaining SQ after October or (b) pineal-mediated photic information earlier in, or before, SQ is more important than immediate information.

(1) Hearn, J.P. J. Reprod. Fert. 39, 235-241 (1974).
(2) Tyndale-Biscoe, C.H. and Hawkins, J. In Reproduction and Evolution (J.H. Calaby & C.H. Tyndale-Biscoe, eds). Aust. Acad. Sci. Canberra. pp. 245-252 (1977).
(3) Sadleir, R.M.F. and Tyndale-Biscoe, C.H. Biol. Reprod. 16, 605-608 (1977).
(4) Renfree, M.B., Lincoln, D.W., Almeida, O.F.X. and Short, R.V. Nature 392, 138-139 (1981).
(5) Kennaway, D.J., Gilmore, T.A., and Seamark, R.F. Endocr. 110, 1766-1772 (1982).

Appendix E.2

MELATONIN PROFILE CHANGES IN THE TAMMAR WALLABY.

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During the season of natural increase in daylength the corpus luteum (CL) and blastocyst of Tammars are held in quiescence. When animals on long daylength (LD 15:9) were changed abruptly to LD 12:12 the CL resumed activity and births occurred 29-36 days later. The pineal may mediate this stimulatory photic information, being reflected in changes of the diurnal profile of plasma melatonin. To test this four female adult wallabies were exposed to LD 15:9 (days -40 to -1) then LD 12:12 (days 0 to +5) and blood samples taken 2 hourly (dark) and 4-6 hourly (light) on days -4, 0, +5. One hourly samples were also taken for 4 hours after dark on days 0 to +5. The mean nocturnal rise in melatonin (pg/ml. $\pm$ s.e.m.) was significantly higher ($P<0.05$) on day +5 (134, $\pm$ 12.4), than on both day 0, (74.4 $\pm$ 13.6) and day -4, (74, $\pm$ 12.1). A significant elevation above mean light levels (32.4 $\pm$ 6.0) was first detected 2 hours after dark on day 0 (84.5 $\pm$ 7.9) but one hour after dark on day +5, (96.5 $\pm$ 12). Births were recorded on days +31, +32, +32 and +33, so blastocyst reactivation had occurred by day +6. These changes in amplitude and duration of the nocturnal rise of melatonin may be of the magnitude or timing necessary to initiate blastocyst reactivation.

COMPARISON OF THE EFFECTS OF SUPERIOR CERVICAL GANGLIONECTOMY AND PINEALECTOMY ON SEASONAL QUIESCENCE IN THE TAMMAR WALLABY.

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Denervation of the pineal gland by superior cervical ganglionectomy (SCGX) is employed as a simpler alternative to pinealectomy (PINX) but as different effects of these treatments on serum prolactin are reported for the rat (1) a comparative study of these procedures on photosensitive seasonally breeding mammals is warranted.

In the tammar SCGX in March to May abolishes seasonal quiescence (SQ) after August (2), but PINX in October did not abolish SQ and blastocyst reactivation occurred at the normal time in December and January (3). Differences in these results could be due to a seasonal response to loss of pineal function or to differences inherent in the two surgical procedures. To test this a group of tammars was subjected to SCGX (n = 7) or Sham SCGX (n = 5) in October and the results compared to the results of PINX already reported (3). The integrity of surgery was confirmed by pre- and post-operative melatonin profiles, tyrosine hydroxylase levels of excised tissue and the manifestation of ptosis.

Births or oestrus were recorded 80.7 ± 7.4 SEM days (SCGX) and 55.4 ± 14.5 SEM days (Sham SCGX) after surgery ($P > 0.05$, $t = -1.687$ (Table 1). The time to birth/oestrus following SCGX was not significantly different to that reported following PINX (87.8 ± 4.93 SEM days; $P > 0.05$, $t = -0.72$).

Table 1. Days to birth/oestrus following SCGX or PINX in SQ (October)

Treatment	n =	Days to birth/oestrus post-op.	MEAN ± SEM
SCGX	7	75, 75, 104, 66, 87, 106, 52	80.7 ± 7.4
Sham SCGX	5	104, 26, 73, 37, 37,	55.4 ± 14.5
PINX	5	97, 92, 77, 98, 75,	87.8 ± 4.93
Sham PINX	6	113,134, 104,105, 98, 98,	108.67± 5.55

The results indicate that SCGX in SQ has the same effect as PINX at this time and both differ from the effect of SCGX performed before SQ (in March to May). This confirms that there is a seasonal difference in response of the tammar to the pineal gland.

(1) Cardinali, D.P., Faigón, M.R., Scacchi, P., and Moguilevsky, J. *J. Endocr.* 82, 315-319 (1979)
(2) Renfree, M.B., Lincoln, D.W., Almeida, O.F.X. and Short, R.V. *Nature* 392, 138-139 (1981)
(3) McConnell, S.J., and Hinds, L. *Proc.Aust.Physiol.Pharmacol.Soc.* 13(2), 173P (1982).

Appendix E.4

PINEAL GLAND FUNCTION IN THE TAMMAR WALLABY - (BOLLIGER AWARD PAPER)

S.J. McConnell, Behavioural Biology, Australian National University.

During the season of natural increase in daylength the corpus luteum (CL) and blastocyst of Tammars are held in quiescence. When animals on long daylength (LD 15:9) were changed abruptly to LD 12:12 the CL resumed activity and births occurred 29-36 days later. The pineal may mediate this stimulatory photic information, being reflected in changes of the diurnal profile of plasma melatonin.

To test this, four female adult wallabies were exposed to LD 15:9 (days -40 to -1) then LD 12:12 (days 0 to +14) and blood samples taken 2 hourly (light) on days -4,0,+5. One hourly samples were also taken for 4 hours after dark on days 0 to +5. The mean nocturnal rise in melatonin (pg/ml $\pm$ s.e.m.) was significantly higher ($P < 0.05$) on day +5 (134 $\pm$ 12.4), than on both day 0, (74.4 $\pm$ 13.6) and day -4 (74 $\pm$ 12.1). A significant ($P < 0.05$) elevation above the mean light level of day -4 (19.95 + 3.26) was first detected 2 hours after dark on day 0 (84.5 $\pm$ 15.8) but one hour after dark on day +5 (96.5 $\pm$ 24).